CHARACTERIZATION OF THE TEMPERATURE DEPENDENT CATABOLISM OF 2,4'-DICHLOROBIPHENYL BY A POLYCHLORINATED BIPHENYL DEGRADING PSYCHROTOLERANT *Hydrogenophaga* SP. IA3-A

ADEWALE JIBOWU LAMBO

Your file Votre référence
ISBN: 978-0-494-42110-9
Our file Notre référence
ISBN: 978-0-494-42110-9

Characterization of the Temperature Dependent Catabolism of 2,4'-Dichlorobiphenyl by a Polychlorinated Biphenyl Degrading Psychrotolerant *Hydrogenophaga* sp. IA3-A

By

©Adewale Jibowu Lambo (B.Sc. Hon., M.Env.Sc.)

A thesis submitted to the school of Graduate Studies in

partial fulfillment of the requirements of the degree of

Doctor of Philosophy

Department of Biology

Memorial University of Newfoundland

September, 2006

St. John's Newfoundland Canada

Abstract

A biphenyl-utilizing bacterium isolated from polychlorinated biphenyls (PCBs)-contaminated soil grew on tryptic soy or biphenyl at 4 to 40 °C and at 5 to 35 °C, respectively. Though the bacterium utilizes selected aromatic compounds as sole carbon and energy source, it only cometabolized chlorinated biphenyls. Analysis of the profile of cellular fatty acids showed that the bacterium is most closely related to *Hydrogenophaga taeniospiralis* (Willems *et al.*, 1989). The gram-negative rod, tentatively identified as *Hydrogenophaga* sp. IA3-A, formed yellow colonies on nutrient agar and denitrified nitrate to nitrogen. The optimal temperature, pH, and substrate concentration for oxidation of 2,4'-dichlorobiphenyl (2,4'-diCB) in 0.05 M sodium phosphate buffer was 30 °C, 7.5, and 0.25 mM, respectively. The extracts of cells grown on biphenyl or in the presence of 2,4'-diCB contained the activity of biphenyl-2,3-dioxygenase (BPDO).

The bacterium degraded mostly mono- to trichlorinated biphenyls (i.e. monoCBs, diCBs, and triCBs) and few tetrachlorinated biphenyls in commercial mixtures. The extents of removal of many of the PCBs were similar at 5 ° and 30 °C. The PCBs that were removed in Aroclor 1221, Aroclor 1232, and Aroclor 1248 was dependent on the composition of the PCB mixtures as well as the number and pattern of chlorination of the congeners.

Chlorinated benzoates (CBAs) were end-products of transformation of 2,3-, 2,4'-diCBs, and 2,4,4'-triCB but cells accumulated significant levels of 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoic acids (HOPDAs) from 2,4'-diCB and 2,4,4'-triCB. The formation of HOPDAs from PCB mixtures or 2,4-diCB and 2,4,4'-triCB indicates that the bacterium preferentially attacked PCBs at the rings bearing *para*-chlorine. This could

account for the accumulation of HOPDA from 2,4'-diCB or 2,4,4'-triCB. Further transformation studies at selected temperatures using 2,4'-diCB indicated that although the parent compound was significantly degraded at low or moderate temperatures (10 °, 25 °, 37 °C), excess level of HOPDA(s) accumulated from 2,4'-diCB leading to poor production of 2- and 4-CBA. The degradation of 2,4'-diCB was inhibited at a higher temperature (45 °C), but most of the degraded compound was recovered as CBAs without excess production of HOPDA. Trimethylsilyl-derivatized culture extracts contained multiple isomeric intermediates with mass spectra that are similar to those of monochlorohydroxybiphenyls, monochlorodihydroxybiphenyls, dichlorodihydrodiols, dichlorodihydroxybiphenyls, and dichlorinated HOPDAs. The detection of some of these products in culture extracts could not be accounted for on the basis of the previously proposed routes of transformation 2,4'-diCBs. Nonetheless, it is suggested that multiple pathways are used for the transformation of 2,4'-diCB and that the pathways are regulated differently at different temperature. This novel findings is the first reported case in which production of excess level of potentially toxic HOPDA from 2,4'-diCB could be avoided under specific conditions in a bacterium that preferentially attacked *para*-substituted rings of PCBs.

Three chromatographic fractions from crude cell extracts separated using a Fast-Flow DEAE Sepharose column were required together to restore maximum activity of partially purified BPDO. The enzyme was specific for (chloro)biphenyls but it shows less activity against 2,4,4'- and 2,2',4-triCBs. BPDO was active against benzene, toluene, and xylenes, and it retained activity against (chloro)biphenyls at 7 °C, but not against 4-CBA or benzene. It was stable at 0 ° to 63 °C and its activity is enhanced in the presence of 1

μM FAD. Unlike other metal ions, mercury (II) and copper (II) severely inhibited the activity of BPDO. The optimal pH, temperature, and substrate concentration for BPDO activity in air-saturated 50 mM MES buffer was 6.0, 25 ^{o}C, and 150 μM, respectively. The apparent K_m of partially purified BPDO for biphenyl was 77.5 μmol and the V_{max} value for NADH was 0.04 μmol per min^{-1} mg^{-1} protein. The activity of BPDO was inhibited in the presence of excess biphenyl.

List of Original Papers

Lambo, A.J., and Patel, T.R. (2006). Isolation and characterization of a biphenyl-utilizing psychrotolerant bacterium, *Hydrogenophaga taeniospiralis* IA3-A, that cometabolize dichlorobiphenyls and polychlorinated biphenyl congeners in Aroclor 1221. J. Basic Microbiol. **46**: 94-107.

Lambo, A.J., and Patel, T.R. (2006). Cometabolic degradation of polychlorinated biphenyls at low temperature by psychrotolerant bacterium *Hydrogenophaga* sp. IA3-A. Curr. Microbiol. **53**: 48-52.

Lambo, A.J. and Patel, T.R. (2006). Biodegradation of polychlorinated biphenyls in Aroclor 1232 and production of metabolites from 2,4,4'-trichlorobiphenyl at low temperature by psychrotolerant *Hydrogenophaga* sp. IA3-A. J. Appl. Microbiol. (Accepted: 22nd of August, 2006).

Lambo, A.J. and Patel, T.R. (2007). Temperature-dependent biotransformation of 2,4'-dichlorobiphenyl by psychrotolerant *Hydrogenophaga* sp. IA3-A: higher temperatures prevents excess accumulation of problematic *meta*-cleavage products. Lett. Appl. Microbiol. **44**: 447-453.

Acknowledgment

I am grateful to God for giving me the patience, the strength, and the perseverance to carry out the research presented in this thesis in spite of all the challenges. My gratitude goes to my supervisor Dr. T. R. Patel for his support and guidance, and also to the members of the supervisory committee, Dr. M. Colbo and Dr. A. Martin, for their useful input into the successful completion of this work.

There are no words to describe my appreciation for the immeasurable support provided by my wife, Atinuke. I cannot forget the succour provided by my lovely daughter, Oluwadamilola, and my budding son, Temidayo. It is hard to imagine what my world would be like at this point without you all in my life. This work wouldn't have been possible without the noble sacrifices of my mother, as well as the inspiration provided by my father, an achiever I could look up to. I cherish the support and prayer offered by my siblings, Sherifat, Adetola, Bukola, and Opeyemi. I owe you all. A big thank you goes to my "mummy Ikorodu" for instilling in me, as far back as my high school years, the desire to get a doctorate. I am indebted to the Oyefeso's, and to all my friends who have sown a seed in this "journey".

Finally, I am grateful for the financial assistance provided by the Department of Biology, the office of the Dean of Science, and the School of Graduate Studies. I appreciate the support of the staff of the Department of Biology, as well as the Staff of the Department of Fisheries and Ocean during the analysis of my samples. I am also using this opportunity to express my gratitude to Debbie for providing the soil samples. You all would remain green in my memory of St. John's, Newfoundland.

Table of Contents **page**

List of Tables **page**

List of Figures **page**

List of abbreviations

BPDO, biphenyl-2,3-dioxygenase

CBA, chlorinated benzoic acid or chlorobenzoate

DecaCB, decachlorobiphenyl

DiCB, dichlorobiphenyl

ECL, equivalent chain lengths

EDTA, ethylenediaminetetraacetic acid

GC, gas chromatography

GC-MS, gas chromatography-mass spectrometry

HeptaCB, heptachlorobiphenyl

HexaCB, hexachlorobiphenyl

HOPDA, 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoic acid

MonoCB, monochlorobiphenyl

NonoCB, nonachlorobiphenyl

OctaCB, octachlorobiphenyls

PCB, polychlorinated biphenyl

PentaCB, pentachlorobiphenyl

ppm, parts per million

SI, similarity index

SIM, selective ion monitoring

TetraCB, tetrachlorobiphenyl

TIC, total ion chromatogram

TLC, thin-layer chromatography

TMS, N-O-bis-(trimethylsilyl)-trifluoroacetamide containing 1% trimethylchlorosilane

TriCB, trichlorobiphenyl

TSA, trypticase soy agar

TSB, trypticase soy broth

Chapter 1 Literature review

1.1 General

Although, the current inventory of world's biodiversity is incomplete, it is estimated that there are 5000 known species of viruses; 4760 known species of bacteria; 69000 known species of fungi; and 40,000 known species of algae (Bull *et al.*, 1992). These represent, approximately, seven percent of the estimated total species of these groups of organisms (Bull *et al.*, 1992). The recent application of molecular techniques to the study of microbial diversity has revealed the existence of an incredible variety of genotypes and species in all known habitats. For example, analyses of the microbial diversity of soils indicate that soils harbor in the order of 7000 different taxa at an abundance of approximately 10^9 cells cm^{-3} (Kassen and Rainey, 2004).

The microbial world is characterized by an incredible metabolic and physiological versatility that allows microorganisms to inhabit hostile ecological niches and to exploit, as carbon and energy sources, compounds unpalatable for higher organisms. This metabolic versatility is evident in their ability to degrade and to grow at the expense of any organic material, which is the basis of the recycling of recalcitrant organic matter in the biosphere (Timmis et al., 1994). The range of organic and inorganic compounds that microorganisms could utilize or transform includes a variety of aromatic and non-aromatic compounds, monomeric and polymeric compounds, metals, and radionuclides.

Carbon is the cornerstone element supporting the perpetuation of life on Earth (Ogunseitan, 2005). In this regard, microbial degradation of aromatic compounds is important to the Earth's carbon cycle (Dagley, 1971). Next to the glucosyl residues, the

benzene ring is the most widely distributed unit of chemical structure in the biosphere, and to a large extent the continuous operation of the carbon cycle depends upon its fission by microorganisms. Most of the aromatic compounds exist in the form of lignin, a plant aromatic biopolymer that is more abundant than protein (Dagley, 1987). Certain group of microorganisms can produce oxygenases that are capable of inserting oxygen in the benzene nucleus, therefore, making it amenable to degradation and utilization as a carbon and energy source (Gibson and Subramanian, 1984).

The introduction and accumulation of anthropogenic xenobiotic compounds in the environments has significant consequences for the earth's carbon cycle. Elevated concentrations of anthropogenic compounds have also been found in remote and hostile environments, including the Arctic (Bright et al., 1995a, 1995b). The accumulation of highly toxic and recalcitrant pollutants in the environment highlights the fact that microbial infallibility is not adequate to protect the biosphere from industrial activities. The novelty of these compounds indicates that microorganisms have not had enough time to evolve appropriate pathways to degrade them (Timmis et al., 1994). However, studies have shown that many of these anthropogenic compounds could be mineralized by microorganisms, serving as source of carbon, sulfur, or nitrogen. But in some cases, degradation of the compounds is incomplete, as they are only partially transformed into intermediates that may be more toxic than the original compound (Reineke, 1984).

In the present study, background information on microbial degradation of aromatic compounds, in particular, chlorinated aromatics is presented in order to illustrate the diversity of metabolic options that are available for the conversion of xenobiotic compounds within microbial cells. An overview of aromatic compounds degradation by cold-adapted

microorganisms is also presented. Thereafter, this study attempts to extend the boundary of the current knowledge of a halogenated aromatic compound (2,4'-dichlorobiphenyl) degradation by a cold-adapted bacterial strain.

1.2 Microbial Degradation of Benzenoid Compounds

1.2.1 Lignin

About 25 % of the Earth's biomass is composed of compounds that have a benzene ring as the main structural constituent. Green plants synthesize a major proportion of these aromatic compounds and assemble them to form lignin, a complex polymer of irregular structure that is stabilized by a complexity of ether and other linkages lacking in readily hydrolyzable bonds (Fig. 1.1) (Gibson and Harwood, 2002; Glazer and Nikaido, 1995). Most lignin is found within the cell walls, where it is intimately interspersed with hemicelluloses, forming a matrix that surrounds the orderly cellulose microfibrils and serving as a barrier against microbial attack (Kirk and Farrell, 1987; Jong et al. 1994a).

Lignin is formed by free-radical-mediated copolymerization of three precursor alcohols: *p*-hydroxycinnamyl (coumaryl) alcohol, which gives rise to *p*-hydroxyphenyl units in the polymer; 4-hydroxy-3-methoxycinnamyl (coniferyl) alcohol, which is the guaiacyl units; and 3,5-dimethoxy-4-hydroxycinnamyl alcohol, that is referred to as the syringyl units. Most gymnosperm lignins contain guaiacyl units. Angiosperm lignins contain approximately equal amounts of guaiacyl and syringyl units. Both types of lignin contain only small amounts of *p*-hydroxyphenyl units (Kirk and Farrell, 1987).

Lignin is primarily defined as a 600 – 1000 kd molecule that is too big to enter cells. Thus, the structural feature of lignin is a constraint on the biodegradative systems that is responsible for the initial attack of the macromolecule. These constraints require that biodegradation of lignin must be extracellular, non-specific, and non-hydrolytic. Lignin is not biodegraded anaerobically (Kirk and Farrell, 1987). Many bacteria have been reported to aerobically degrade or mineralize a wide variety of (^{14}C-lignin) lignocellulose

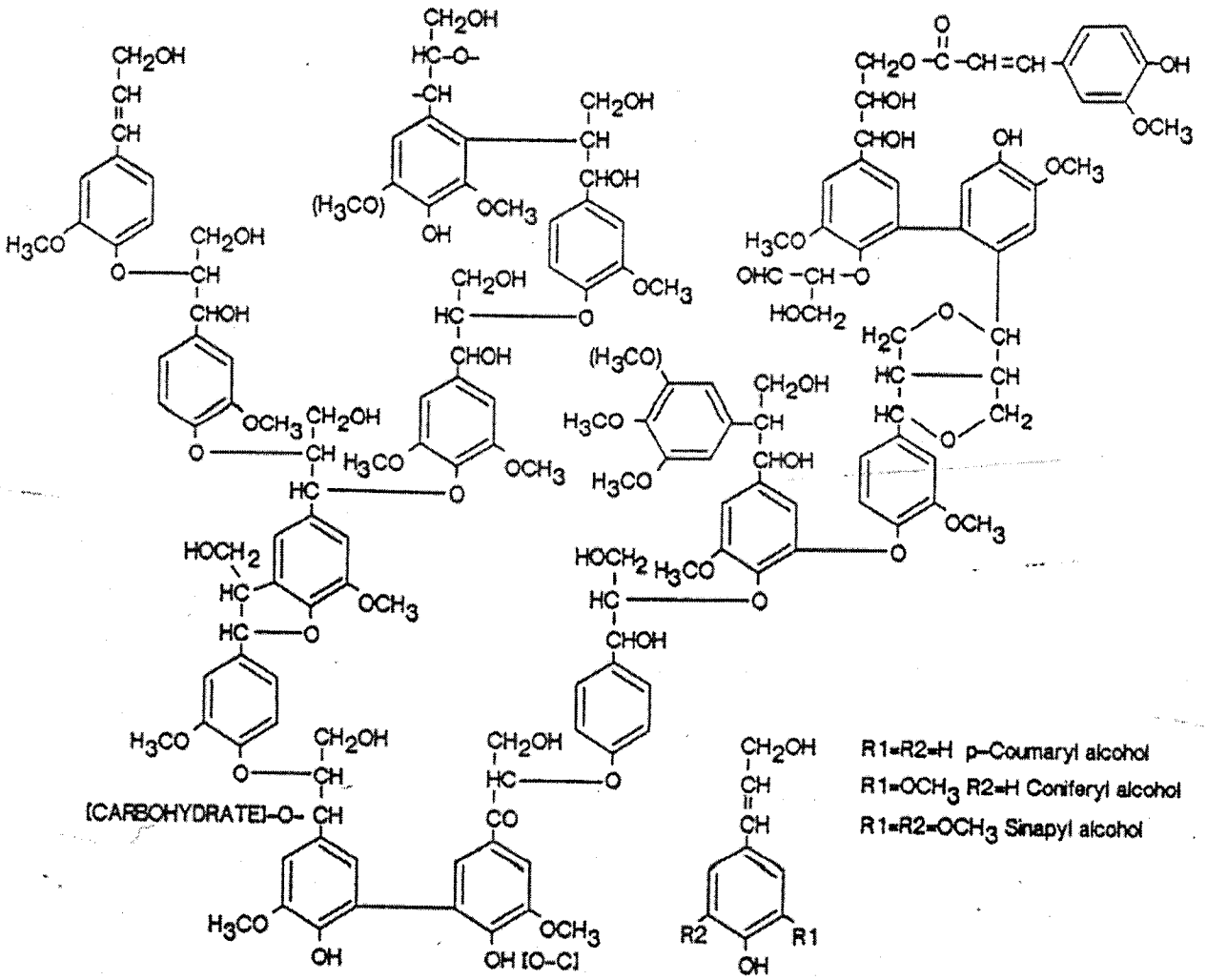


Fig. 1.1. Schematic structural formula for lignin. The three precursor alcohols of lignin are shown at the lower right corner (Kirk and Farrel, 1987).

as well as oligomeric ^{14}C-labeled synthetic lignin (Vicuna, 1988). Bacteria exhibiting ligninolytic activity includes tunneling-bacteria (Daniel et al., 1987; Singh et al., 1987) and several actinomycetes (Crawford, 1978). However, these bacteria have not been shown to degrade polymeric lignin or lignified tissues in wood. Extensive degradation of lignin under aerobic conditions is limited to certain fungi, particularly the white-rot fungi (Kirk and Farrell, 1987). Even in the white-rot fungi, lignin cannot be degraded as a sole source of carbon and energy, and its degradation only occurs in the presence of other readily utilizable co-substrates like hemicellulose and cellulose (Jong et al., 1994a). White-rot wood decay that is accompanied by substantial loss of lignin has been reported for few species of ascomycetes that includes *Xylaria, Libertella,* and *Hypoxylon* (Kirk and Farrell, 1987). Lignin degrading non-white-rotting basiodiomycetes includes *Cyathus stercoreus* and several other *Cyathus* species (Abott and Wicklow, 1984; Wicklow et al. 1984), as well as brown-rotting species capable of limited aromatic hydroxylation and ring cleavage and extensive demethylation of aromatic methoxy groups (Kirk, 1984). The ability of the brown-rot fungi to mineralize the backbone lignin polymer is limited (Kirk and Farrell, 1987).

The white-rot basidiomycetes degrade lignin more rapidly and extensively than any other microbial group that has been studied. Similar to the brown-rot fungi, the white-rot fungi invade the lumen of wood cells prior to secretion of the enzymes that are capable of degrading lignin and other wood components. The white-rot fungus produces extracellular lignin-modifying enzymes (Kirk and Farrell, 1987). The best characterized lignin-modifying enzymes are laccase, lignin peroxidases (LiPs), and manganese peroxidases (MnPs) (Hatakka, 1994). The most studied white-rot fungus, *Phanerochaete chrysosporium*, does not produce laccase (Kirk and Farrell, 1987). The other enzymes involved in lignin degradation

in some white-rot fungi include hydrogen peroxide-producing glyoxal oxidase, aryl alcohol oxidase, and veratyl alcohol oxidase (Kersten, 1990; Muheim, et al., 1990; Sannia et al., 1991). The initial process of ligninase oxidation of lignin termed "enzymatic combustion" (Kirk and Farrell, 1987) is a non-specific reaction that is predominantly a one-electron, or two-electron oxidation of lignin model compounds to produce unstable cation-radicals which undergo a variety of non-enzymatic sequential reactions that are responsible for the large number of degradation intermediates formed from lignin as it is degraded by white-rot fungi (Fig. 1.2) (Higuchi, 1987). As observed in the burning of lignin that its conversion to carbon dioxide and water is thermodynamically favored, the depolymerization of lignin is kinetically favored because ligninases oxidize their substrates by one electron reactions. In biological systems, however, the oxidative mineralization of organics unlike combustion normally takes a channelled route to thermodynamically stable end products (Kirk and Farrell, 1987).

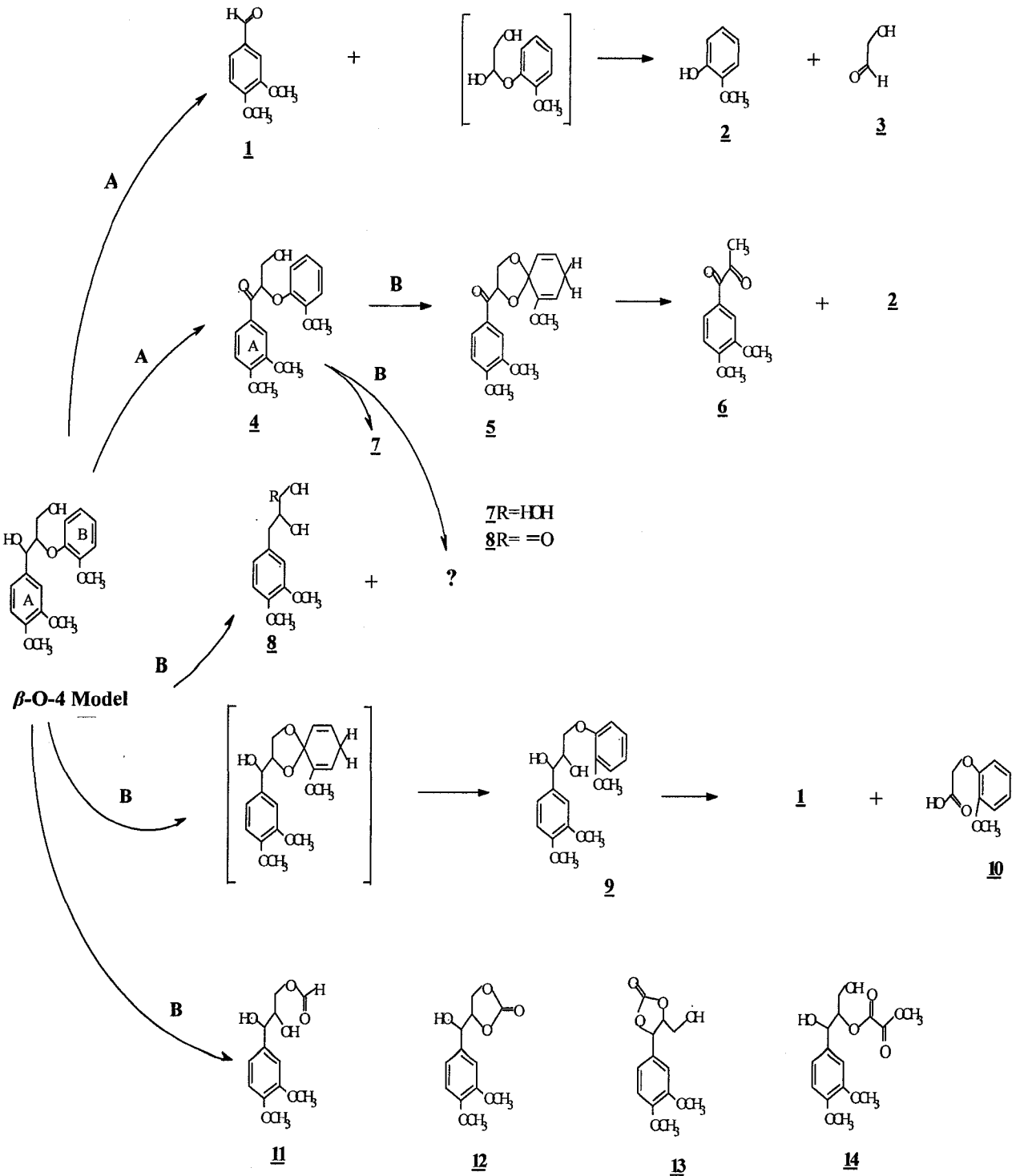


Fig. 1.2. Products of oxidation of *β*-O-4 model compounds by ligninase-H_2O_2. Oxidation of the compound can be initiated at ring *A* or ring *B* as shown (Kirk and Farrell, 1987).

1.2.2 Aromatic and Polycyclic Aromatic Compounds

Aerobic transformation or mineralization of a variety of substituted and unsubstituted aromatic compounds by diverse groups of microorganisms is widely reported. *Pseudomonas putida* mt-2 and *Rhodococcus rhodochrous* strain OFS grew on toluene as sole source of carbon and energy (Assinder and Williams, 1990; Vanderberg et al., 2000). *Pseudomonas putida* F1 utilized benzene as sole source of carbon and energy (Gibson et al., 1974). Six marine bacterial strains belonging to the *Roseobacter* group grew on benzoate, anthranilate, salicylate, *p*-hydroxybenzoate, vanillate, coumarate, ferulate, and protocatechuate (Buchan et al., 2000). Actinomycetes bacteria *Arthrobacter auresecens* TW17 and *Nocordia* sp. strain TW2 that are capable of growth on benzene, nitrobenzene, *p*-cresol, and phenol also degraded *p*-nitrophenol (Hanne et al., 1993). Cell-free manganese peroxidase system from *Nematoloma frowardii* partially mineralized pentachlorophenol, dinitrophenol, and catechol (Hofrichter et al., 1998), whereas *P. chrysosporium* substantially mineralized benzene, toluene, ethylbenzene, and xylenes under non-ligninolytic conditions (Yadav and Reddy, 1993). Deuteromycete fungus, *Cladophialophora* sp. strain T1 cometabolized xylenes, and utilized toluene and ethylbenzene as sole carbon and energy sources (Prenafeta-Boldu, 2002). Eukaryotic alga *Ochromonas danica* produced carbon-dioxide from phenol and incorporated phenol carbon into biomass (Semple and Cain, 1996).

Aerobic biodegradation of polycyclic aromatic hydrocarbon (PAH) compounds is widespread among microorganisms. Several bacterial and fungal strains mineralized PAH compounds that include naphthalene, methylnaphthalene, phenanthrene, fluoranthene, or pyrene (Boochan et al., 2000; Churchill et al., 1999; Deziel et al., 1996; Grund et al., 1992; Stringfellow and Aitken, 1995). Naphthalene, phenanthrene, fluorene, pyrene, and

benzo(a)pyrene were cometabolized or biotransformed in some bacterial, algal, and fungal strains (Narro et al., 1992; Stringfellow and Aitken, 1995; Sutherland et al., 1990; Wunder et al., 1994). Dibenzothiopene, a polycyclic aromatic sulfur heterocycle compound, was used as a sole source of carbon, sulfur, and energy by *Brevibacterium* sp. DO (Afferden et al., 1990).

Aerobic degradation of substituted or unsubstituted aromatic compounds by microbial cells is initiated by oxygenation of the aromatic ring or substituents by oxygen-inserting monooxygenase or dioxygenase enzymes. Monooxygenases catalyzes the incorporation of one atom from oxygen into substrates, and dioxygenases catalyzes the incorporation of both atoms of oxygen into substrates (Harayama et al., 1992). Bacterial degradation of substituted aromatic compounds like toluene and nitrophenol is initiated by different types of (mono)oxygenases and the compounds could be degraded through different pathways (Fig. 1.3 and Fig. 1.4) (Duetz et al., 1994; Spain, 1995). The majority of monooxygenases are single-component enzymes, though multicomponent monooxygenases such as phenol and toluene-4-monooxygenase have been described. Alkyl-group monooxygenases are usually multicomponent and they consist of a hydroxylase component and one or two electron-transport component(s). Aromatic-ring dioxygenases are multicomponent enzymes having a hydroxylase and electron-transport components (Harayama et al., 1992). Oxidation of aromatic substrates by these enzymes and the subsequent reactions results in the formation of dihydroxylated benzene ring (Harwood and Parales, 1996). Thus, many aromatic compounds are metabolically altered to produce three common dihydroxylated intermediates, which are

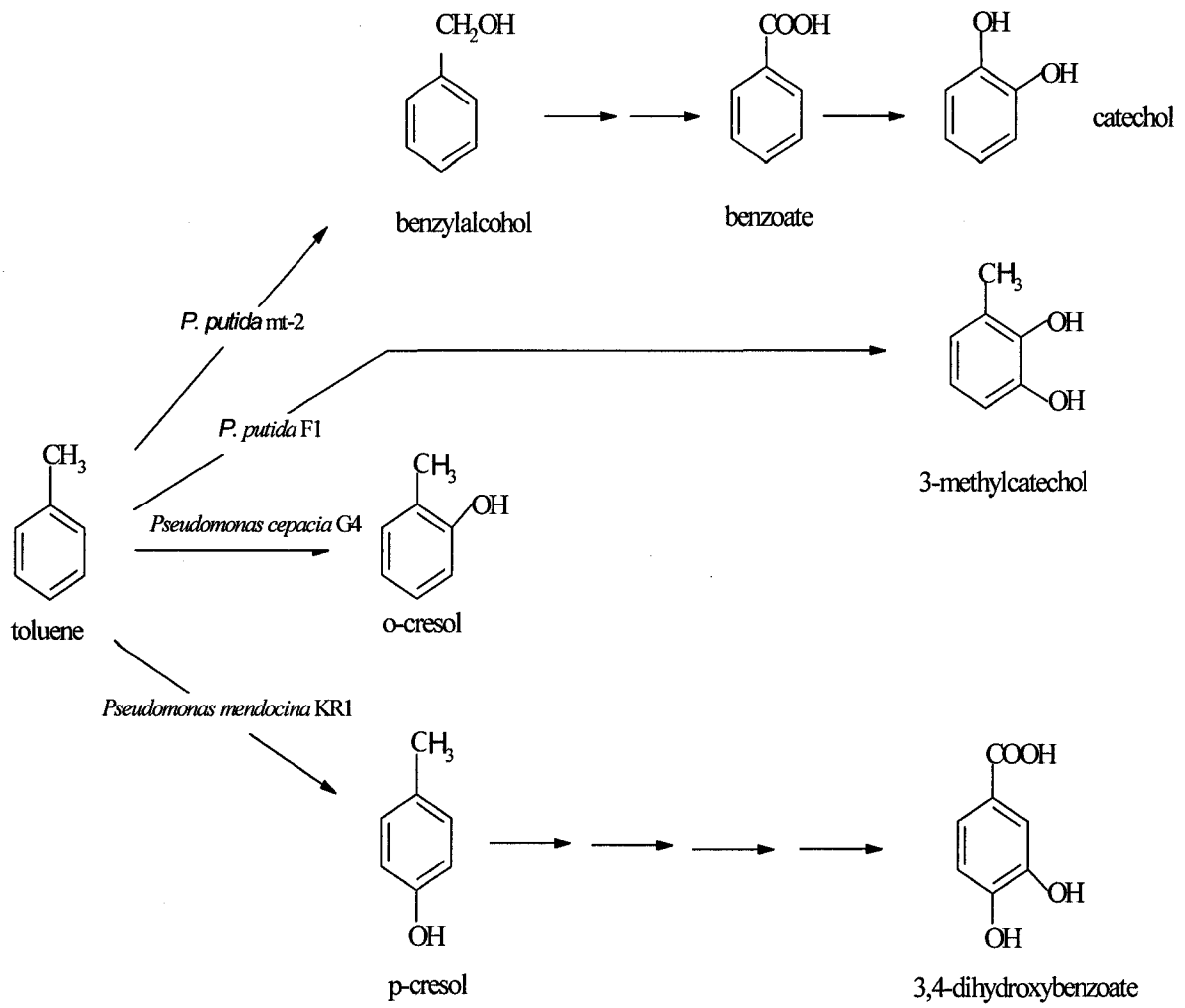


Fig. 1.3. Initial steps of different pathways for the bacterial degradation of toluene (adapted from Duetz et al., 1994). Note: oxidation of toluene by *P. putida* mt-2 is initiated by a monooxygenase at the methyl substituent; oxidation of toluene by *P. cepacia* G4 and *P. mendocina* KR1 is initiated by a monooxygenase at different sites on the aromatic ring; and dioxygenase catalyzed oxidation of toluene by *P. putida* F1 occurs on the aromatic ring.

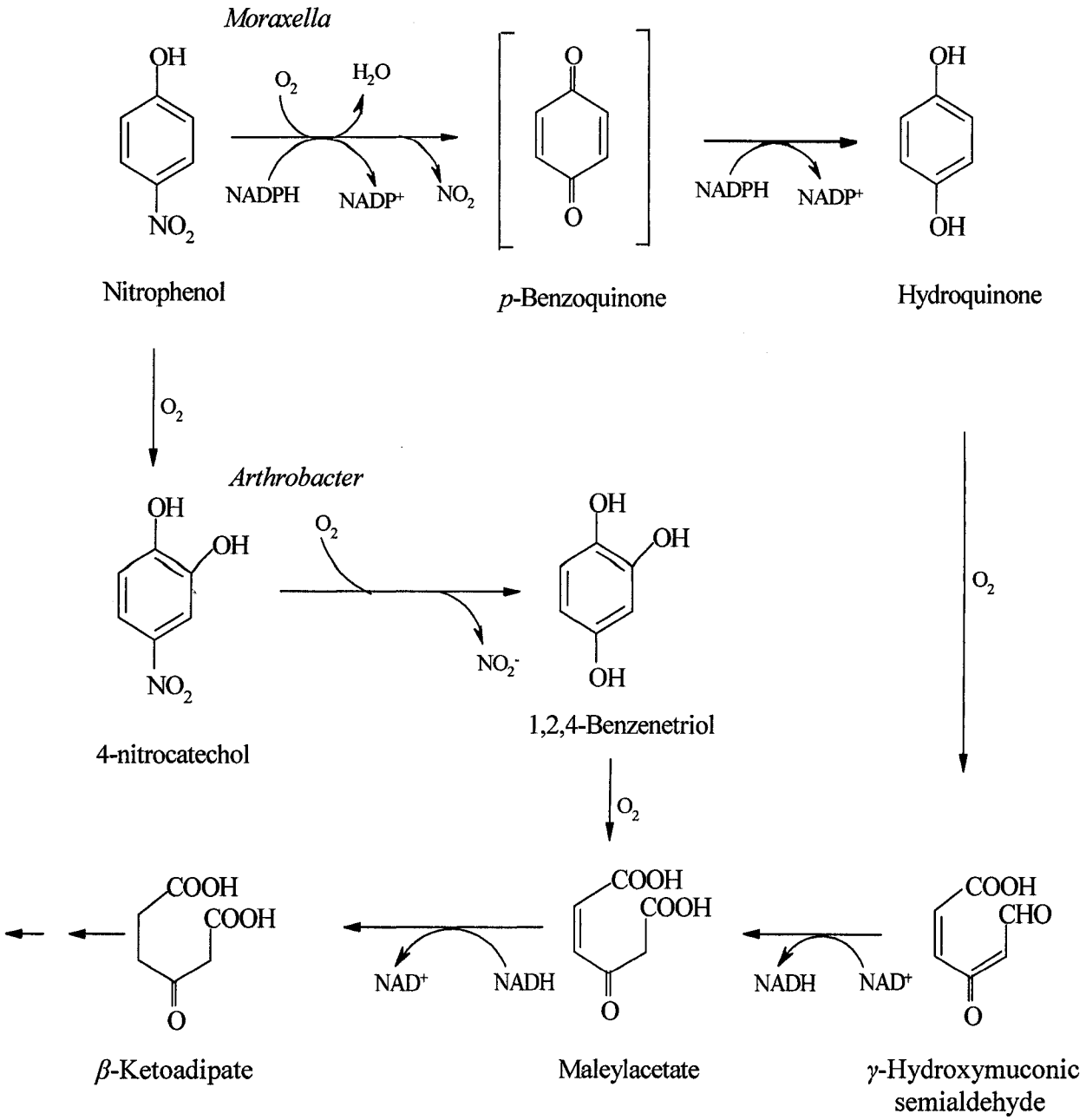


Fig. 1.4. Initial steps and different pathways for degradation of 4-nitrophenol by *Moraxella* sp. and *Arthrobacter* sp. (modified from Spain et al., 1995). Note: biodegradation of 4-nitrophenol by *Moraxella* sp. is initiated by a monooxygenase; and biodegradation of the compound by *Arthrobacter* sp. is initiated by a dioxygenase.

catechol, protocatechuate, and gentisate. The second phase of degradation proceeds by ring fission of the dihydroxylated intermediates followed by subsequent reactions leading to the generation of tricarboxylic acid cycle intermediates. Ring fission is catalyzed by aromatic ring-cleavage dioxygenases and is termed *ortho*-cleavage when it occurs between the hydroxyl groups (i.e. intradiol cleavage) and *meta*-cleavage when it occurs adjacent to one of the hydroxyls (i.e. extradiol cleavage). The *meta*- and *ortho*-cleavage pathways ensure the dissimilation of catechol and protocatechuate (Fig. 1.5). A third pathway, the gentisate pathway is utilized when the two hydroxyl groups on the aromatic ring are *para* to each other and cleavage occurs between the carboxyl-substituted carbon and the adjacent hydroxylated carbon (Harwood and Parales, 1996).

The *ortho*-cleavage of catechol and protocatechuate results, respectively, in the production of *cis,cis*-muconate and β-carboxy-*cis,cis*-muconate (Ornston, 1966), which are further dissimilated through the β-ketoadipate pathway to produce succinate and acetyl-CoA (Fig. 1.6). The *meta*-cleavage of catechol results in the production of formate, pyruvate, and acetaldehyde, whereas the *meta*-cleavage of protocatechuate results in the production of formate and pyruvate as end-products (Ornston and Ornston, 1966). In the gentisate pathway, cleavage of the aromatic ring results in the formation of maleylpyruvate which is isomerized to fumarylpyruvate. The fumarylpyruvate is hydrolyzed to fumarate and pyruvate. Fumarate is converted to L-malic acid, and maleic acid and pyruvate are formed from maleylpyruvate. The maleic acid is subsequently converted into D-malic acid (Fig. 1.7) (Bayla and Barbour, 1984). Substrates that are degraded through the *meta*- and gentisate pathway include phenolics, steroids, alkylbenzoic sulfonates, and polycyclic aromatics. Aromatic compounds

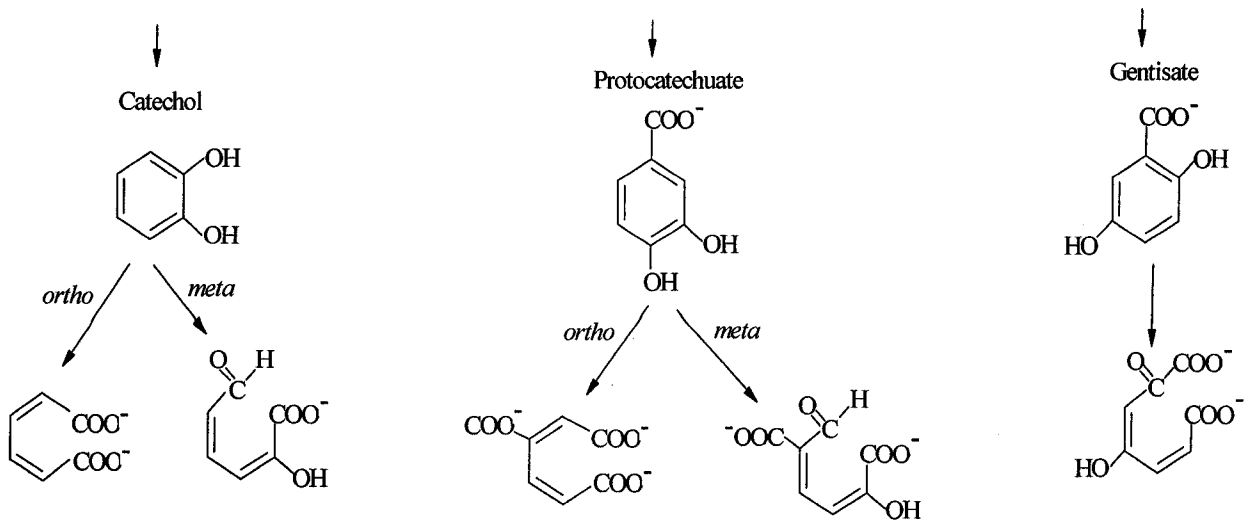


Fig. 1.5. Major aerobic routes of aromatic ring cleavage: *ortho*-, *meta*-, and gentisate cleavage (adapted from Harwood and Parales, 1996). The arrow above catechol, protocatechuate, and gentisate indicates that these compounds are produced as common intermediates from parent aromatic compounds.

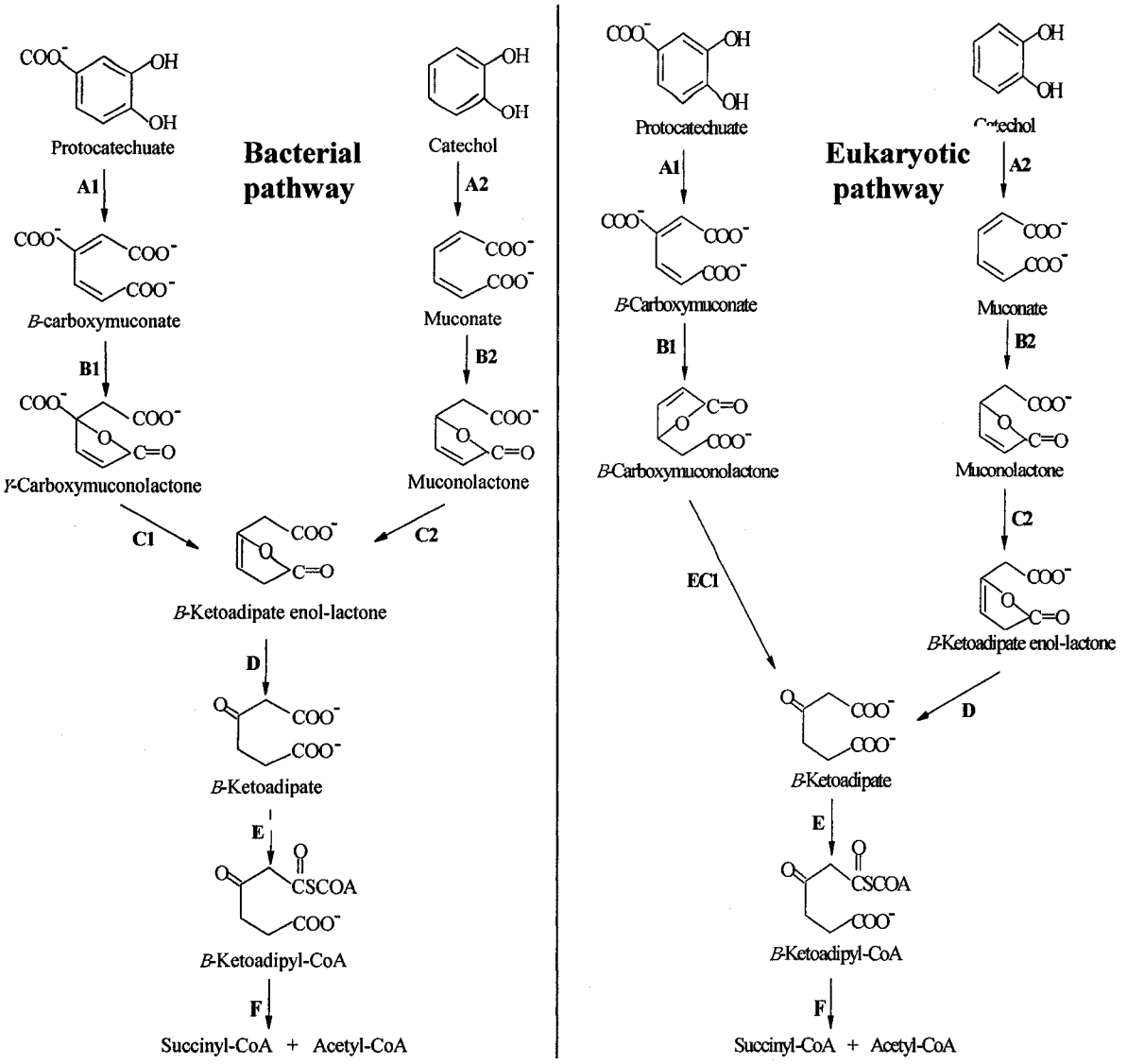


Fig. 1.6. Bacterial and Eukaryotic pathways for dissimilation of catechol and protocatechuate. Enzymatic steps: A1, protocatechuate 3,4-dioxygenase; B1, β-carboxy-*cis,cis*-muconate lactonizing enzyme; C1, γ-carboxymuconolactone decarboxylase; EC1, β-carboxymucono-lactone decarboxylase; A2, catechol 1,2-dioxygenase; B2, *cis,cis*-muconate lactonizing enzyme; C2, muconolactone isomerase; D, enol-lactone hydrolase; E, β-ketoadipate succinyl-CoA transferase; F, β-keto-adipyl-CoA thiolase (adapted from Harwood and Parales, 1996).

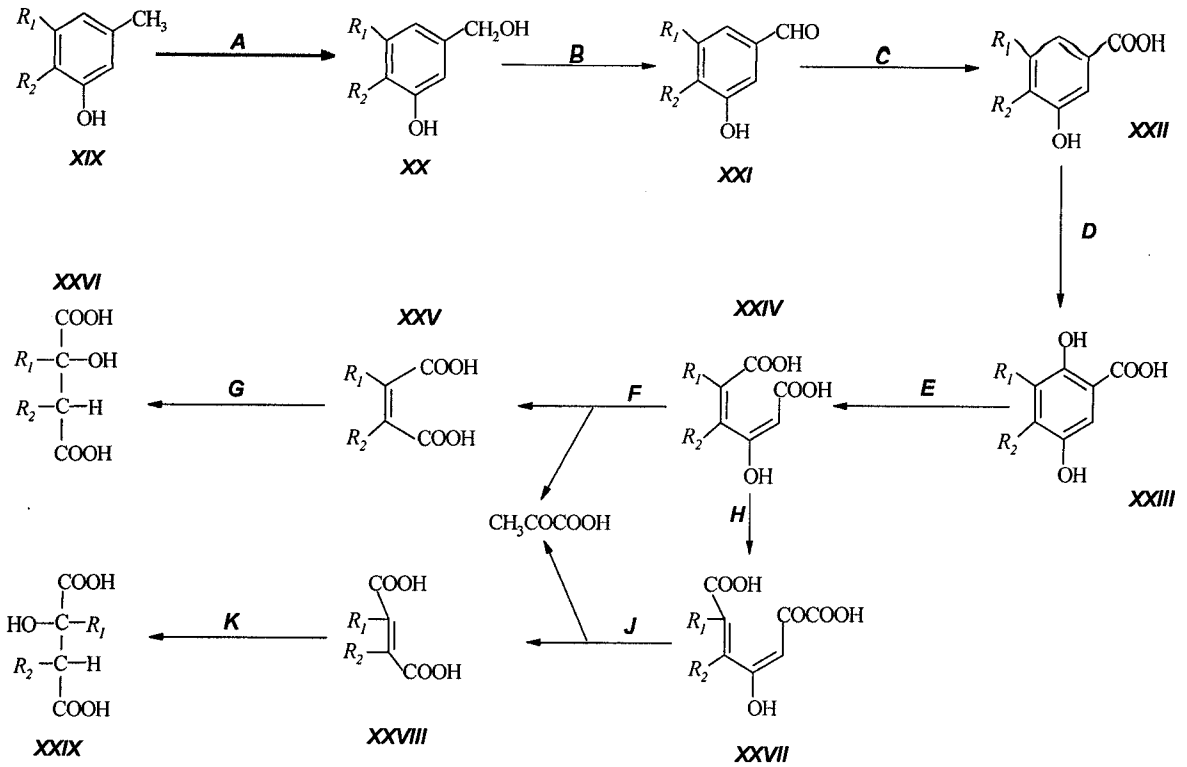


Fig. 1.7. The gentisate pathway for the degradation of *m*-cresol, and 2,5- and 3,5-xylenol (adapted from Bayly and Barbour, 1984). **Compounds: R_1 = H, R_2 = CH_3**; XIX, *m*-cresol; XX, 3-hydroxybenzylalcohol; XXI, 3-hydroxybenzaldehyde; XXII, 3-hydroxybenzoate; XXIII, gentisate, XXIV, maleylpyruvate; XXV, maleic acid; XXVI, D-malic acid; XXVII, fumarylpyruvate; XXVIII, fumarate; XIX, L-malic acid; XIX, 2,5-xylenol; XX, 3-hydroxy-4-methylbenzylalcohol; XXI, 3-hydroxy-4-methylbenzaldehyde; XXII, 3-hydroxy-4-methylbenzoate; XXIII, methylgen-tisate; XXIV, 5-methylmaleypyruvate; XXV, citraconic acid; XIX, 3,5-xylenol; XX, 3-hydroxy-5-methylbenzylalcohol; XXI, 3-hydroxy-5-methylbenzaldehyde; XXII, 3-hydroxy-5-methylbenzoate; XXVIII, 3-methyl-gentisate; XXIV, 6-methylmaleylpyruvate; XXV, citraconic acid. Enzymes: A, methylhydroxylase; B, alcohol dehydrogenase; C, aldehyde dehydrogenase; D, 6-monooxygenase; E, gentisate 1,2-dioxygenase; F, maleylpyruvate hydrolase; G, maleate hydratase; H, isomerase; J, fumarylpyruvate hydrolase; K, fumarase

such as *m*-cresol, 3-hydroxybenzoic acid, β-naphthol, salicyclic acid, anthranilic acid, and xylenol are specifically degraded through the gentisate pathway. (Bayla and Barbour, 1984; Chapman, 1972).

The *meta*-fission pathway enzymes differ from those of the *ortho*-cleavage pathway in their ability to catalyze the degradation of methylated aromatic hydrocarbons like toluene and xylene. The *meta*-cleavage pathways for the degradation of some aromatic compounds are plasmid encoded. In contrast, the *ortho*-cleavage pathways are almost always chromosomally encoded, and it often coexists in bacteria with plasmid-encoded *meta*-pathways (Harwood and Parales, 1996). The catechol and protocatechuate *ortho*- and *meta*-cleavage pathway also exhibits some level of difference in the mode of enzyme induction, number of substrate inducers, and substrate specificity (Bayly and Barbour, 1984; Stanier and Ornston, 1973). Additionally, a wider spectrum of aromatic compounds that are obligatory to enzymes with broader substrate specificity and induction patterns are channeled through the *meta*-pathway (Bayla and Barbour, 1984).

The β-ketoadipate pathway has not yet been reported in the members of the Archea, however, the eukaryotic version has been identified in members of the ascomycetous and basidiomycetous yeasts and fungi. The catechol branch of the eukaryotic pathway is present in some but not all eukaryotes, and the protocatechuate branch differs from that of prokaryotes in that β-carboxy-*cis*,*cis*-muconate is cyclized to give β-carboxymuconolactone rather γ-carboxymuconolactone (Fig. 1.6). The two branches of the fungal β-ketoadipate pathway converge at β-ketoadipate rather than β-ketoadipate enol lactone, as in the bacterial pathway (Harwood and Parales, 1996).

The initial reaction in the degradation of PAHs in prokaryotes and eukaryotes is catalyzed by oxygenases. Prokaryotes utilize dioxygenases to incorporate two atoms of oxygen into PAHs to form *cis*-dihydrodiol. The eukaryotic monooxygenase cytochrome P-450 system catalyzes the incorporation of one atom of oxygen into the compounds to produce arene-oxides (Gibson and Subramanian, 1984). In the case of naphthalene, the *cis*-naphthalene dihydrodiol is converted to 1,2-dihydroxynaphthalene, which undergoes ring-cleavage to form *cis*-o-hydroxybenzalpyruvate, followed by isomerization to form *trans*-o-hydroxybenzalpyruvate. Both isomers are enzymatically converted to salicylaldehyde and pyruvate. The salicylaldehyde is transformed by a dehydrogenase to salicylate which undergoes an oxidative decarboxylation to form catechol (Gibson and Subramanian, 1984). The catechol may be cleaved by *ortho*- or *meta*-fission as described previously (Fig. 1.8). Alternatively, some bacterial strains may convert salicylate to gentisate rather than catechol. The gentisate is further degraded via the gentisate pathway (Fuenmayor et al., 1998). The arene oxides produced from PAHs by eukaryotic monooxygenases could isomerise spontaneously by NIH shift to form phenols or it could be converted by epoxide hydrases to form a *trans*-dihydrodiol compound (Fig. 1.9). In addition, glutathione may react non-enzymatically with arene oxides, or enzymatically in a reaction catalyzed by soluble glutathione-*S*-epoxide transferase. However, studies indicate that epoxide hydrase may be more important than glutathione in detoxifying arene oxides (Jerina and Daly, 1974). Arene oxides can also bind a variety of nucleophiles, including cellular macromolecules such as DNA, RNA, or protein. The toxic, carcinogenic, and mutagenic effects of aromatic compounds often correlate with the extents of this binding. Thus, arene oxides are strongly implicated as causative agents in producing these effects (Jerina and Daly, 1974).

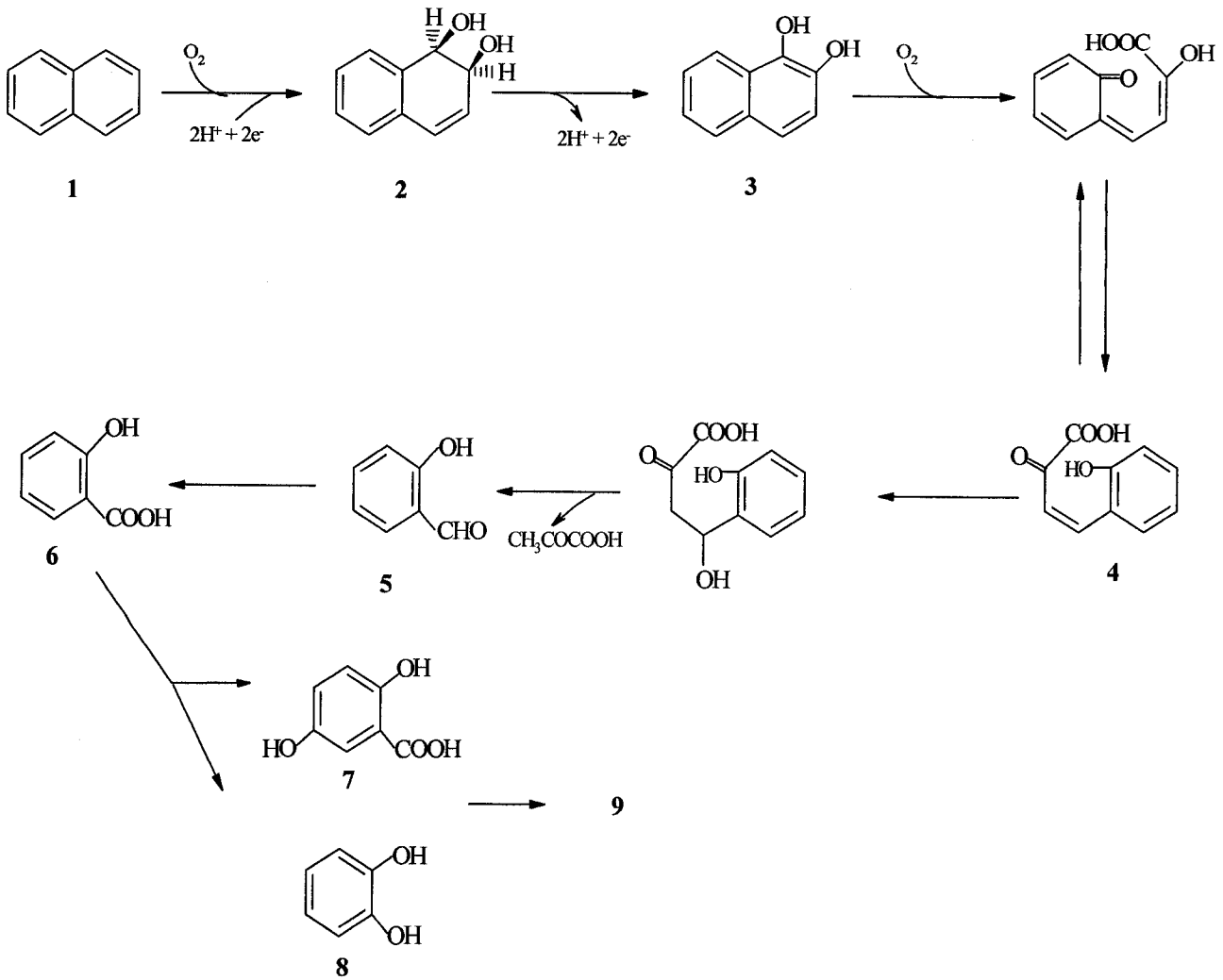


Fig. 1.8. Proposed pathway for bacterial degradation of naphthalene (adapted from Gibson and Subramanian, 1984). Compounds: 1, naphthalene; 2, *cis*-naphthelene dihydrodiol; 3, 1,2-dihydroxynaphthalene; 4, *cis*-o-hydroxybenzalpyruvate; 5, salicylaldehyde; 6, salicyclic acid; 7, gentisic acid; 8, catechol; respective ring fission products.

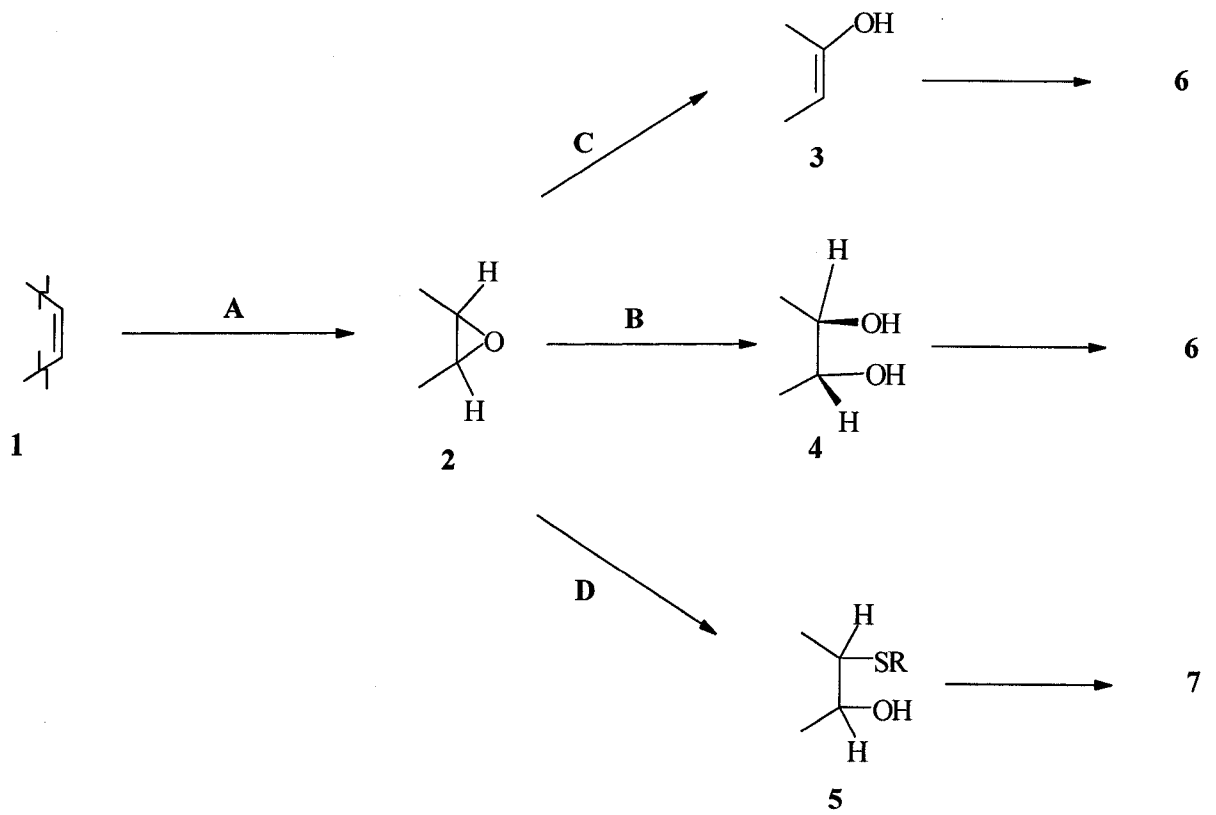


Fig. 1.9. Transformation reactions of aromatic hydrocarbons by mammals (adapted from Gibson and Subramanian, 1984). Compounds: 1, aromatic hydrocarbons; 2, arene oxide; 3, phenol; 4, *trans*-dihydrodiol; 5, glutathione conjugate; 6, sulfates and glucuronides; 7 mercapturic acids. A, cytochrome P-450; B, epoxide hydratase; C, NIH shift; D, glutathione transferase.

1.2.3 Anaerobic Degradation of Aromatics and Polycyclic Aromatics

Anaerobiosis usually occurs in habitats in which the supply of oxygen is exceeded by its consumption and this is typical in many natural environments that include flooded soils, sediments, landfills, lagoons, anaerobic fresh and ocean waters and some groundwater (Berry *et al.*, 1987). It is known that compounds such as benzene, toluene, ethylbenzene, and all the three xylene isomers can be biodegraded in the absence of oxygen by a broad diversity of microorganisms. These compounds have been shown to serve as carbon and electron sources for bacteria growing phototrophically or respiratorily with nitrate, manganese, ferric iron, sulfate, and carbon dioxide as electron acceptors (Chakraborty and Coates, 2004).

One of the conditions required for complete mineralization of organic compounds in anoxic ecosystems by anaerobic microorganisms is the necessity for microbial food chains or synthrophic associations. Studies have shown that three major groups of microorganisms, the fermenters, proton reducers, and methanogens, are essential for complete mineralization of organic compounds in the absence of light and under low oxygen conditions in sites that are low in electron acceptors other than carbondioxide (Berry *et al.*, 1987). For example, a phenol and 4-hydroxybenzoate mineralizing consortium consisting of a short and long rod-shaped bacterium and low numbers of *Desulfovibrio* cells grew only in syntrophy with methogenic archeabacterium, *Methanospirillum hungatei* (Knoll and Winter, 1989).

Prior to the work carried out by Vogel and Grbic-Galic (1986), it was generally accepted that anaerobic metabolism of benzenoid compounds could only proceed through reductive pathways (Evans, 1977). However, it has been suggested that benzenoid compounds could also be metabolized anaerobically through an oxidative route by incorporation of the oxygen from water into aromatic rings (Vogel and Grbic-Galic, 1986).

Anaerobic photometabolism of benzoate typified by the purple non-sulfur bacterium, *Rhodopseudomonas palustris*, proceed by hydrogenation of the aromatic ring followed by hydration and ring cleavage (Fig. 1.10) (Dutton and Evans, 1969). A significant aspect of this study is a proposal that reduction of aromatic compounds in *R. palustris* involves a reductase that is coupled to a low-redox-potential component (e.g. ferredoxin) of the light-induced electron transport system (Dutton and Evans, 1969). Light-induced mineralization of several aromatic compounds that include benzoate, benzyl alcohol, vanillate, and syringate by phototrophic *Rhodomicrobium vanielli* have also been reported (Wright and Madigan, 1991). A similar light-independent reductive pathway is used by *Moraxella* sp. for the degradation of benzoic acid under nitrate respiring condition (Berry *et al.*, 1987). In contrast to these reports, Taylor *et al.* (1970) proposed that anaerobic catabolism of benzoate and *p*-hydroxybenzoate by facultative *Pseudomonas* sp. strain PN-1, under nitrate-reducing condition, is initiated by oxidative incorporation of O_2 from water into the aromatic ring of these compounds, and that the intermediate produced could be converted through the pathway that is postulated for *R. palustris* (Fig. 1.11). It appears that strain PN-1 contained low level of protocatechuate 4,5-dioxygenase activity when grown anaerobically on aromatic compounds, showing that this strain possess an oxygenase enzyme system that does not require oxygen as an inducer (Taylor et al., 1970). Anaerobic oxidation of toluene coupled to the reduction of Fe(III) have also been shown for some members of the genus *Geobacter* (Lovely et al., 1989; Coates and Lovley, 2003). Studies indicates that the initial reaction in the anaerobic degradation of aromatic compounds is determined by the nature of the

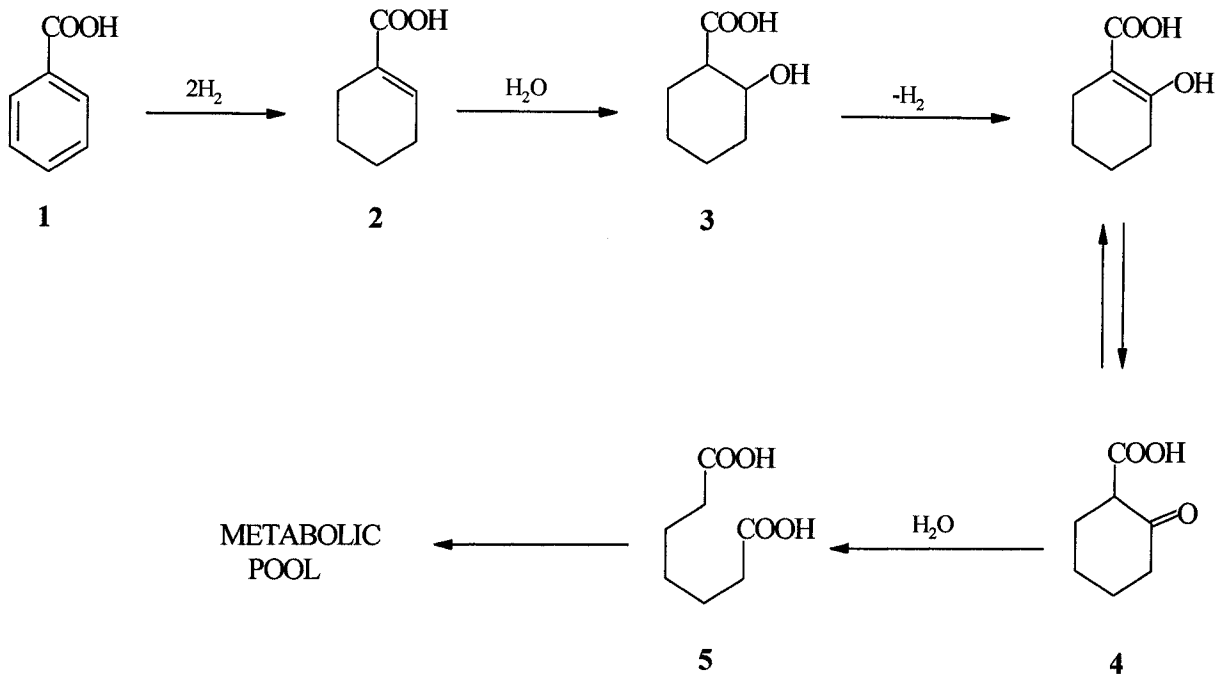


Fig. 1.10. Proposed pathway for photocatabolism of benzoate by *R. palustris* (adapted from Berry *et al.* 1987). Compounds: 1, Benzoate; 2, cyclohex-1-ene-1-carboxylate; 3, 2-hydroxycyclohexane carboxylate; 4, 2-oxocyclohexane carboxylate; 5, pimelate.

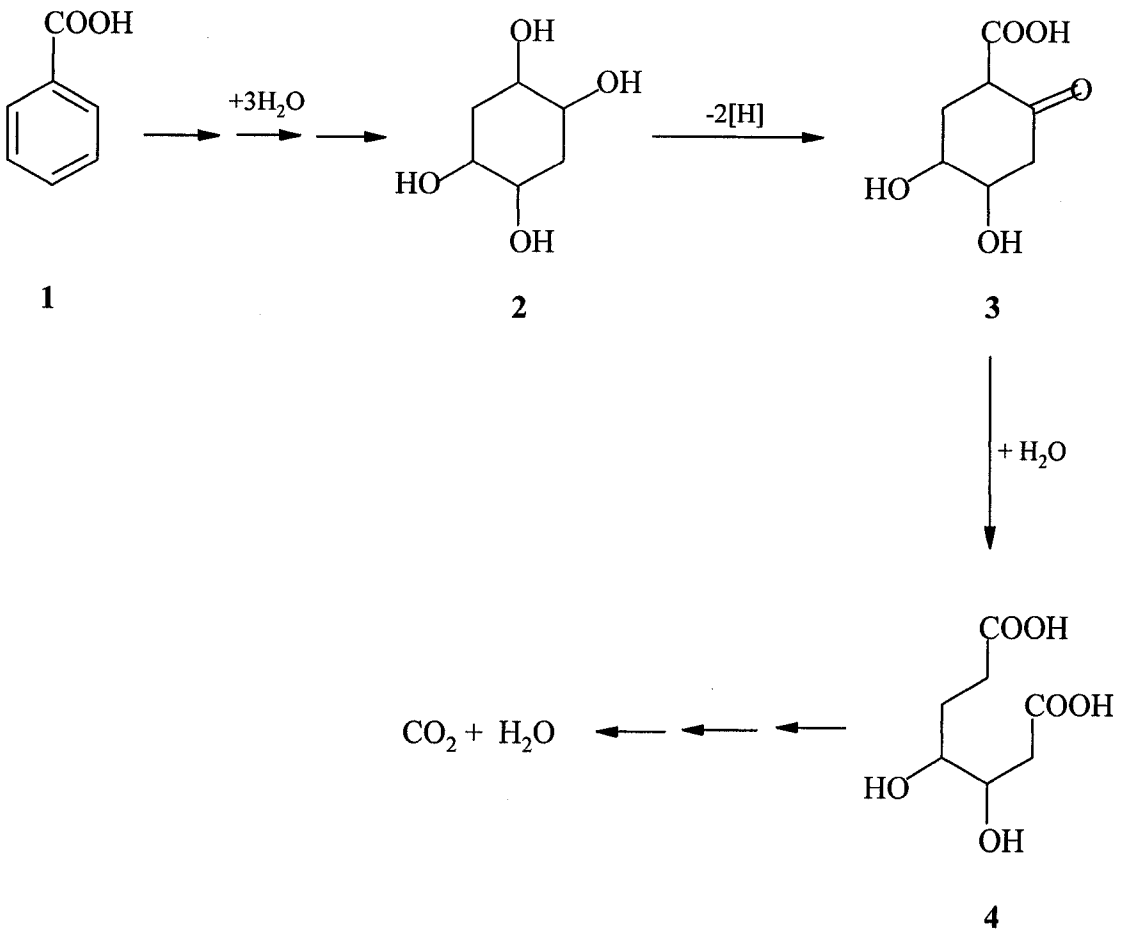


Fig. 1.11. Hypothetical scheme for the saturation of aromatic ring and its subsequent cleavage by *Pseudomonas* PN-1 (adapted from Taylor et al., 1970). Compounds: 1, Benzoic acid; 2, trihydroxycyclohexane carboxylic acid; 3, dihydroxycyclohexan-2-one-1-carboxylic acid; 4, dihydroxy pimelic acid.

substrate as well as the terminal electron acceptor during the reactions (Fig. 1.12) (Chakraborty and Coates, 2004). The central intermediate of these pathways is benzoate or its CoA derivative. The benzoyl-CoA could undergo a ring reduction prior to ring cleavage and oxidation (Chakraborty and Coates, 2004).

Anaerobic biotransformation of nitrogen-substituted aromatic compounds has been shown for bacterial strains belonging to different genera that include *Clostridium*, *Desulfovibrio*, *Bacteroids*, *Haloanaerobium*, *Methanococcus*, and *Pseudomonas* (Spain et al., 1995). In few instances, the nitro compound is used as a source of nitrogen for growth and as terminal electron acceptor (Boopathy and Kulper, 1993; Boopathy and Kulper, 1994). The sequential reduction of 2,4,6-trinitrotoluene (TNT) results in the production of amines, followed by reductive deamination of the aromatic ring. However, evidence supporting the formation of toluene as a by-product of TNT transformation was preliminary and the mechanism involved was not fully understood (Spain et al., 1995). The other products of reductive transformation of TNT in bacteria include aminodinitrotoluene, diaminonitrotoluene, and triaminotoluene (Spain et al., 1995).

In contrast to higher molecular weight PAH compounds, there are several reports of biodegradation of naphthalene under methanogenic, nitrate-reducing, or sulfate-reducing conditions. The mineralization of naphthalene and phenanthrene by sulfidogenic consortia has been reported as well (Zhang and Young, 1997). Anaerobic degradation of naphthalene or phenanthrene is thought to proceed by carboxylation of the aromatic ring to form 2-naphthoate or phenanthrenecarboxylic acid, respectively (Zhang and Young, 1997). However, a study by Bedessem et al. (1997) suggests an alternate route of naphthalene degradation with naphthalenol as a possible intermediate.

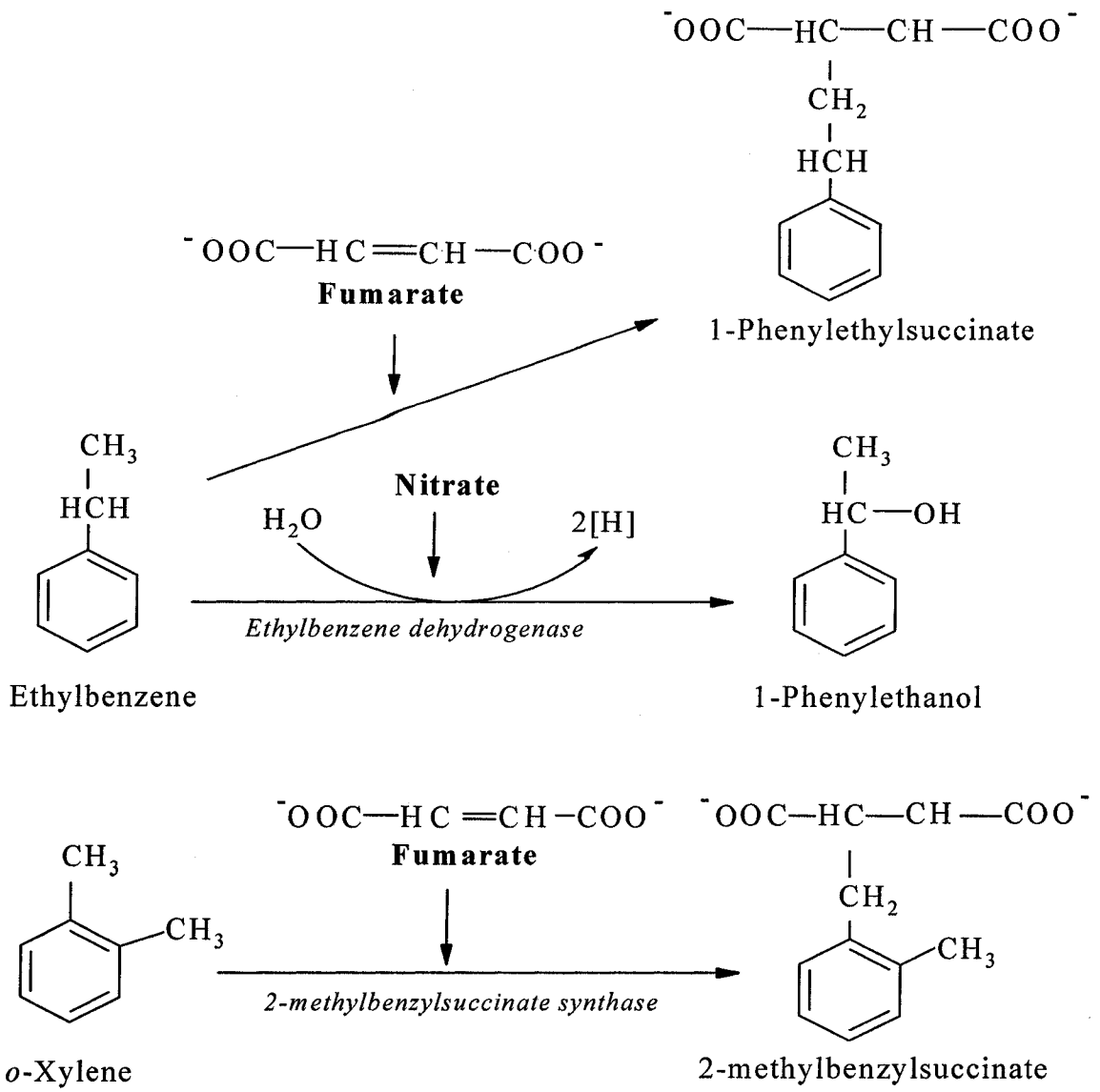


Fig. 1.12. Initial reactions in the anaerobic degradation of aromatic compounds under different electron accepting conditions. The name of the compounds in bold indicates the electron accepting substrate (adapted from Chakraborty and Coates, 2004).

1.2.4 Degradation of Halogenated Aromatic Compounds

Halogenated organic compounds are produced industrially in large quantities and they represent an important class of environmental pollutants. In addition, haloorganic compounds are produced in abundance naturally (Fetzner, 1998). Abiotic sources of organo-halogenated compounds include volcanic activity, and forest and brush fires, which are known to produce polychlorinated dibenzodioxin and polychlorinated dibenzofuran (Gribble, 1994). More than 2000 organohalogenated chemicals are released into the biosphere by various marine organisms, higher plants and ferns, insects, bacteria, fungi, and mammals (Gribble, 1994, 1996) Brominated hydrocarbons are predominantly synthesized by marine organisms and chlorinated compounds are mostly produced by terrestrial organisms. In contrast, iodinated compounds occur less frequently, whereas fluorinated compounds are very rare (Gschwend et al., 1985; Gribble, 1994; van Pee, 1996). For example, the white rot fungus *Bjerkendera adusta* produces several volatile organoaromatic compounds include 3-chloro-4-methoxybenzaldehyde and 3-chloro-4-methoxybenzalcohol (Jong *et al.*, 1994b). The hemichordate *Saccoglossus kowaleskii* produces dibromophenol (King, 1996), whereas the bacterium *Streptomyces venezuelae* produces chloramphenicol (Ehrlich *et al.*, 1947). It is now accepted that organohalogens of natural rather than recent anthropogenic origin have primed the development of a broad repertoire of microbial systems to dehalogenate or completely mineralize these compounds, preventing their accumulation in the natural environments (Smidt and de Vos, 2004). The relative novelty of anthropogenically derived halogenated compounds in the environment and the lack of time for microorganisms to evolve the appropriate degradative system are probably responsible for the recalcitrance of many of these compounds (Haggblom, 1990; Reineke and Knackmuss, 1988). Thus, it is a

paradox that all chlorinated organic compounds are persistent and toxic, even though, the vast majority of naturally produced organic chlorine is neither persistent nor toxic (Oberg, 2002).

There are two possible mechanism of aerobic or anaerobic degradation of halogenated aromatic compounds in microorganisms. Cometabolic degradation of haloaromatics occurs when microorganisms catalyze partial transformation of the compounds to end-products that do not support growth. The compounds could also be completely mineralized, serving as a carbon and energy source with subsequent return of the organic halogen to its mineral state (Reineke and Knackmuss, 1988). Cometabolic degradation of haloaromatics usually results in partial return of organic chloride to its mineral state and in many cases the mineral state is not produced. The removal of halogen may occur during the early stage of degradation by reductive, hydrolytic, or oxygenolytic elimination. Alternatively, a non-aromatic halogenated structure may be produced subsequent to the spontaneous lose of halide by hydrolysis, or hydrogen halide by β-elimination (Reineke and Knackmuss, 1988).

Reductive dehalogenation is an important step in the metabolism of highly chlorinated aromatic compounds like pentachlorophenol, hexachlorobenzene, polychlorinated dibenzofuran, polychlorinated dibenzodioxin, and polychlorinated biphenyls (PCBs) (Fetzner, 1998). It has been shown to occur under aerobic or anaerobic conditions. Depending on the substrate, anaerobic reductive dehalogenation may proceed under methanogenic conditions, nitrate-reducing conditions, sulfate-reducing conditions, or through fermentation (Reineke and Knackmuss, 1988). Vargas *et al.* (2000) observed that three fluorobenzoate isomers that were recalcitrant under sulfate-reducing, iron-reducing, and

methanogenic conditions, were depleted within 28 to 84 days under nitrate-reducing conditions.

Anaerobic reductive dehalogenation may be coupled to energy generation or carbon metabolism (Fetzner, 1998). Cometabolic reductive dehalogenation of haloaromatics is less widespread, and it has been shown to occur mainly with haloalkanes (Gopalakrishnan et al., 2003). Reductive dehalogenation coupled to energy generation is termed halorespiration. As revealed by estimation of Gibbs free energy and redox potentials, many halogenated aromatics are excellent electron acceptors in the absence of molecular oxygen, yielding between -130 and -180 kJ mol^- of chlorine removed by hydrogenolytic reductive dehalogenaton. From thermodynamic consideration, based on corresponding redox potential (E'_0) range of +260 and +480 mV, which is much higher than the redox potential of sulfate (SO_4^{2-}/H_2S; E'_0 = -217 mV) and comparable to the redox potential of nitrate (NO_3^-/NO_2^-; E'_0 = +433 mV), it is predicted that reductive dehalogenation should be occurring, though rarely, under aerobic conditions (Smidt and de Vos, 2004). In some cases, halorespiring microorganisms can, by dehalogenation, unlock halogenated compounds for further consumption. *Desulfomonile tiedje* dehalogenated 3-chlorobenzoate to benzoate, which can be mineralized by benzoate-degrading strain using anaerobic pathways described earlier (Smidt and de Vos, 2004). *Dehalococcoides* sp. strain CBDB1 sequentially dehalogenated hexa- and pentachlorobenzene to mixtures of tri- and dichlorobenzene with a growth yield of 2.1 and 2.9 g protein mol^{-1} of Cl^- released (Fig. 1.13) (Gopalakrishnan *et al.*, 2003).

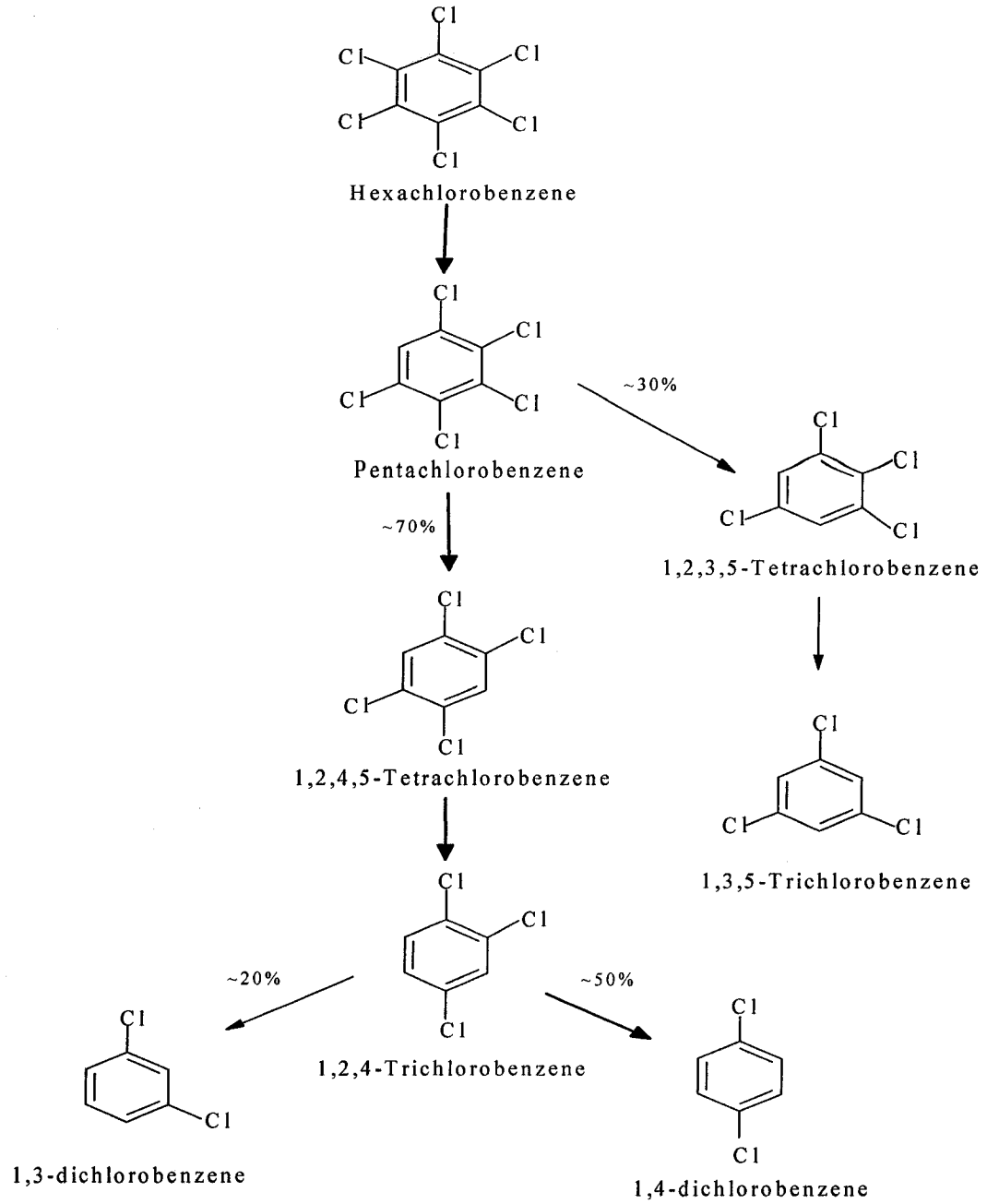


Fig. 1.13. Proposed pathway for hexachlorobenzene and pentachlorobenzene reductive dechlorination by *Dehalococcoides* sp. strain CBDB1 (adapted from Gopalakrishnan *et al.*, 2003). The values indicate the relative amounts of products formation and are related to the total amount of products detected. Bold arrows indicates major dechlorination pathway

Reductive dehalogenation reactions are not restricted to strictly anaerobic bacteria (Fetzner, 1998). Among the haloaromatic dehalogenating enzymes that has been described is a constitutively expressed cofactor-free glutathione-dependent tetrachlorohydroquinone (TCHQ) dehalogenase from *Sphingomonas chlorophenolica* ATCC 39723 that is produced during degradation of pentachlorophenol to chloride, carbon dioxide, and water. After the conversion of pentachlorophenol to TCHQ by pentachlorophenol hydroxylase, TCHQ-dehalogenase catalyzes two successive reductive dehalogenations to convert TCHQ to 2,6-dichloro-*p*-hydroquinone (Fig. 1.14, step 2 and 3) (McCathy et al., 1997). A gluthatione-dependent reductive dehalogenase (LinD) is also involved in the aerobic degradation of cycloaliphatic compound, γ-hexachlorocyclohexane, by *Sphingomonas paucimobilis* UT26 by catalyzing two successive dehalogenation of 2,5-dichlorohydroquinone, an intermediate, to hydroquinone (Miyauchi et al., 1998). Among the intermediates and end-products of degradation of γ-hexachlorocyclohexane are several (chlorinated) aromatic intermediates.

The largest numbers of dehalogenases described are hydrolytic dehalogenases that catalyze nucleophilic displacement reaction with water as the sole cosubstrate (Janssen et al., 1994; Fetzner, 1998). An intermediate step in the degradation of pentachlorophenol is catalyzed by a hydrolytic dehalogenase that convert 2,6-dichloro-*p*-hydroquinone to 6-chloro-1,2,4-trihydroxybenzene (Fig. 1.14, step 4). Aromatic substitution reactions, exemplified by the conversion of 4-chlorobenzoate to 4-hydroxybenzoate (Fig. 1.15), are widespread among several genera of bacteria that includes *Pseudomonas*, *Arthrobacter*, *Acinetobacter*, *Alcaligene*, *Nocordia*, and *Corynebacterium* (Fetzner, 1998). The production of 4-hydroxybenzoate from 4-chlorobenzoate requires three enzymes: which

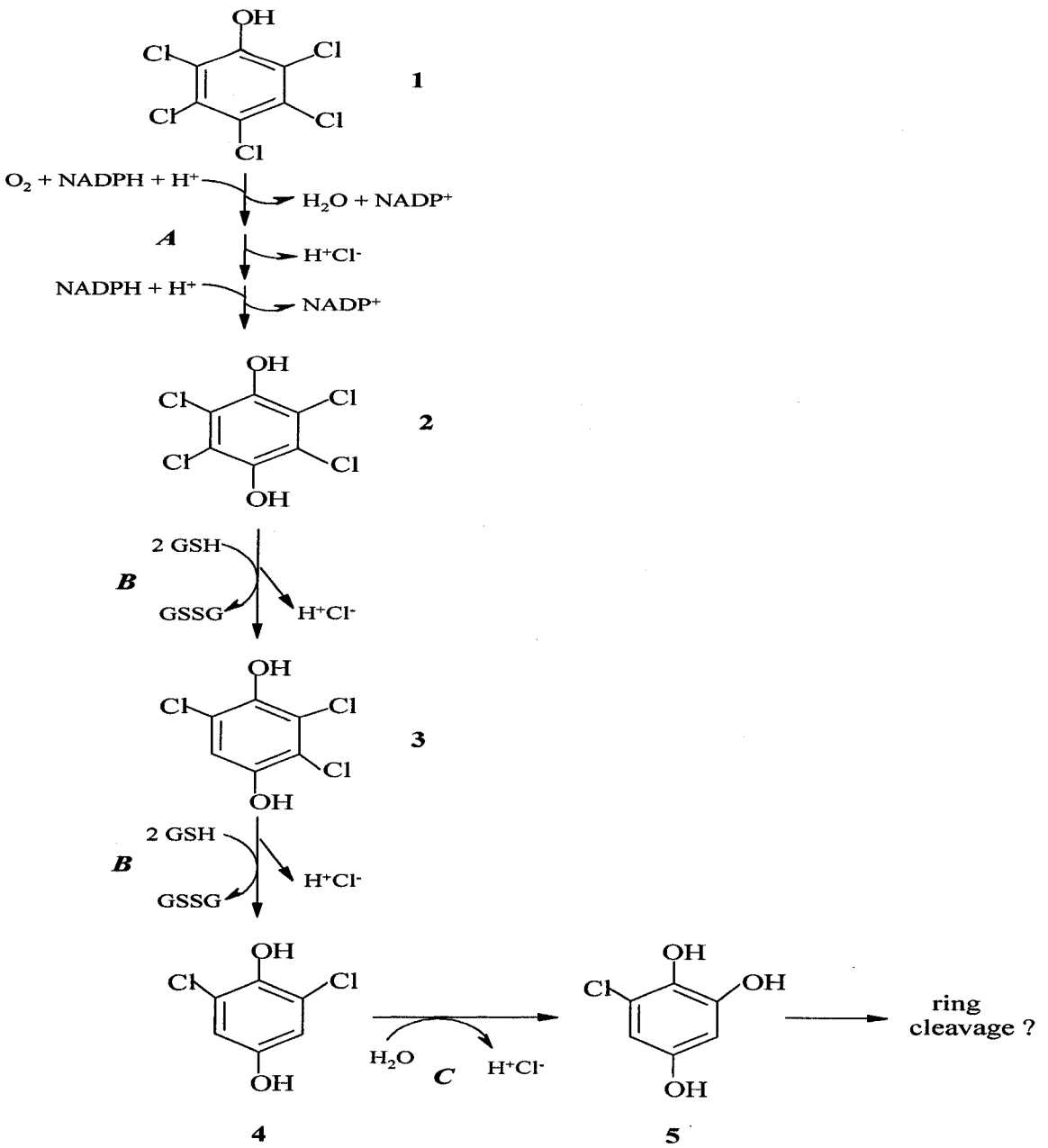


Fig. 1.14. Pathway for pentachlorophenol (PCP) degradation by *S. chlorophenolica* ATCC 39723 (adapted from Fetzner et al., 1998). Compounds: **1**, PCB; **2**, 2,3,5,6-tetrachloro-*p*-hydroquinone; **3**, 2,3,6-trichloro-*p*-hydroquinone; **4**, 2,6-dichloro-*p*-hydroquinone; **5**, 6-chloro-1,2,4-trihydroxybenzene. Enzymes: **A**, PCP monooxygenase (PcpB); **B**, (glutathione-dependent) tetrachlorohydroquinone reductive dehalogenase (PcpC); **C**, 2,6-dichlorohydroquinone chlorohydrolase. *GSH*, glutathione; *GSSG*, oxidized glutathione

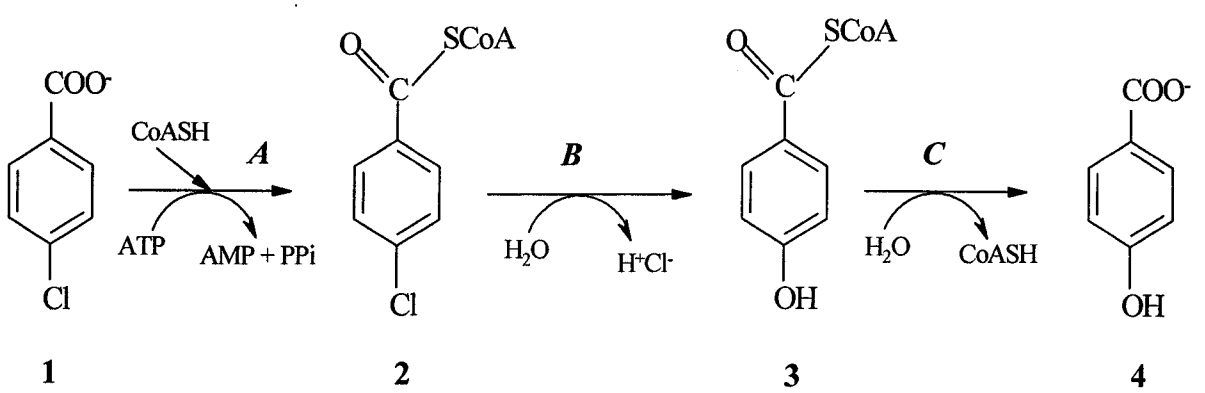


Fig. 1.15. Initial steps of bacterial degradation of 4-chlorobenzoate (adapted from Fetzner et al., 1998). Compounds: **1**, 4-Chlorobenzoate; **2**, 4-chlorobenzoyl-CoA; **3**, 4-hydroxybenzoyl-CoA; **4**, 4-hydroxy-benzoate. Enzymes: **A**, 4-Chlorobenzoate CoA ligase; **B**, 4-chlorobenzoyl-CoA dehalo-genase; **C**, 4-hydroxybenzoyl-CoA thioesterase.

are 4-chlorobenzoate coenzyme A ligase, 4-chlorobenzoyl-CoA dehalogenase, and 4-chlorobenzoyl-CoA thioesterase (Fetzner, 1998). Hydrolytic dehalogenation of 4-iodobenzoate and 4-bromobenzoate has also been demonstrated (Reineke and Knackmuss, 1988).

Oxygenolytic dehalogenation reactions are catalyzed by several monooxygenases and dioxygenases. Cometabolic oxidation occurs when haloaromatics compete with the growth substrate for the active site of oxygenase enzymes (Fetzner, 1998). Therefore, the reaction is often a fortuitous process. The transformation of pentachlorophenol to tetrachlorohydroquinone (Fig. 1.14, step 1) by *S. chlorophenolica* ATCC 39723 is catalyzed by a NADPH-dependent flavoprotein monooxygenase that consumes two mol of NADPH for every mole of PCP that is oxidized (Xun and Orser, 1991). Aerobic degradation of polychlorinated biphenyls is catalyzed by biphenyl-degrading enzymes, and the first reaction is initiated in most bacteria by three-components biphenyl-2,3-dioxygenase that inserts two atoms of molecular oxygen into the *ortho* and *meta* positions of one of the biphenyl rings (Haddock and Gibson, 1995). Dioxygenolytic dechlorination of 2,2'-dichlorobiphenyl, 2,3'-dichlorobiphenyl, and 2,5,2'-trichlorobiphenyl at the 2-*ortho* position is catalyzed by biphenyl-2,3-dioxygenase enzyme from *Pseudomonas* sp. LB400 (Haddock et al., 1995). This enzyme, like other dioxygenases catalyze the formation of *cis*-diols that spontaneously rearomatize leading to elimination of chloride and formation of catechol products (Fig. 1.16) (Fetzner, 1998).

Elimination of halogen can also occur by dehydrodehalogenation, a reaction that is catalyzed by dehydrodehalogenases that eliminate hydrogen chloride (HCl) from their halosubstrates leading to the formation of double bonds. This mechanism is utilized in the

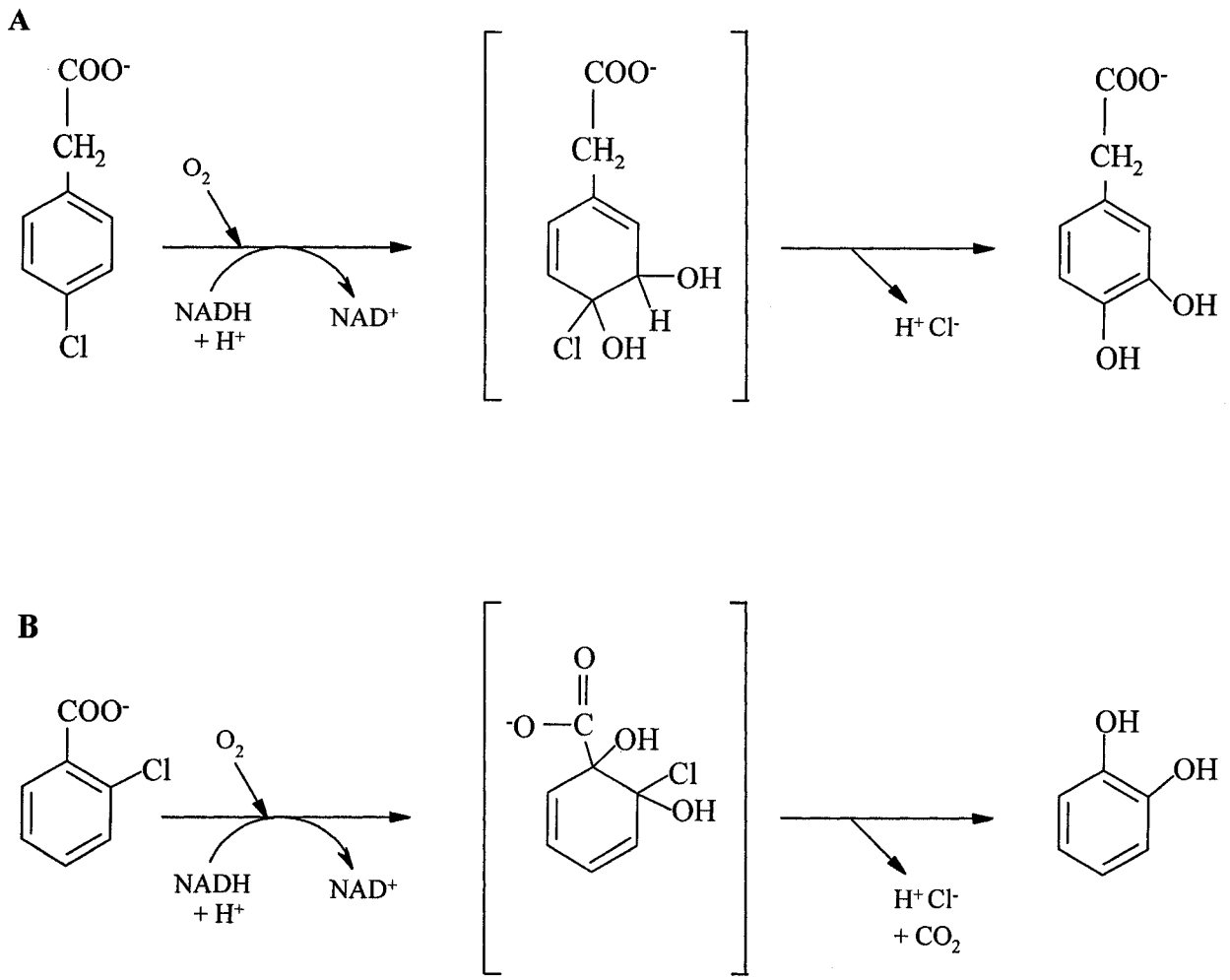


Fig. 1.16. Dioxygenolytic dechlorination of chloroaromatic compounds. **A**, Dioxygenation of 4-chlorophenylacetate by 4-chlorophenylacetate 3,4-dioxygenase from *Pseudomonas* sp. CBS3; **B**, dioxygenation catalyzed by 2-halobenzoate 1,2-dioxygenase from *Pseudomonas aeruginosa* strain 142 (adapted from Fetzner et al., 1998).

modified *ortho*-pathway to eliminate the chloride on the non-aromatic intermediate that is produced after *ortho*-cleavage of chlorocatechols, which are central intermediates in the aerobic metabolism of halogenated aromatic compounds (Fig. 1.17). Cycloisomerization of chlorinated *cis*, *cis*-muconates, the product of *ortho* cleavage of chlorocatechols, result in the formation of dienelactones. Chloromuconate cycloisomerase, unlike muconate cycloisomerase, is able to catalyze the conversion of halogenated substrates to *trans*-dienelactone (Fetzner, 1998).

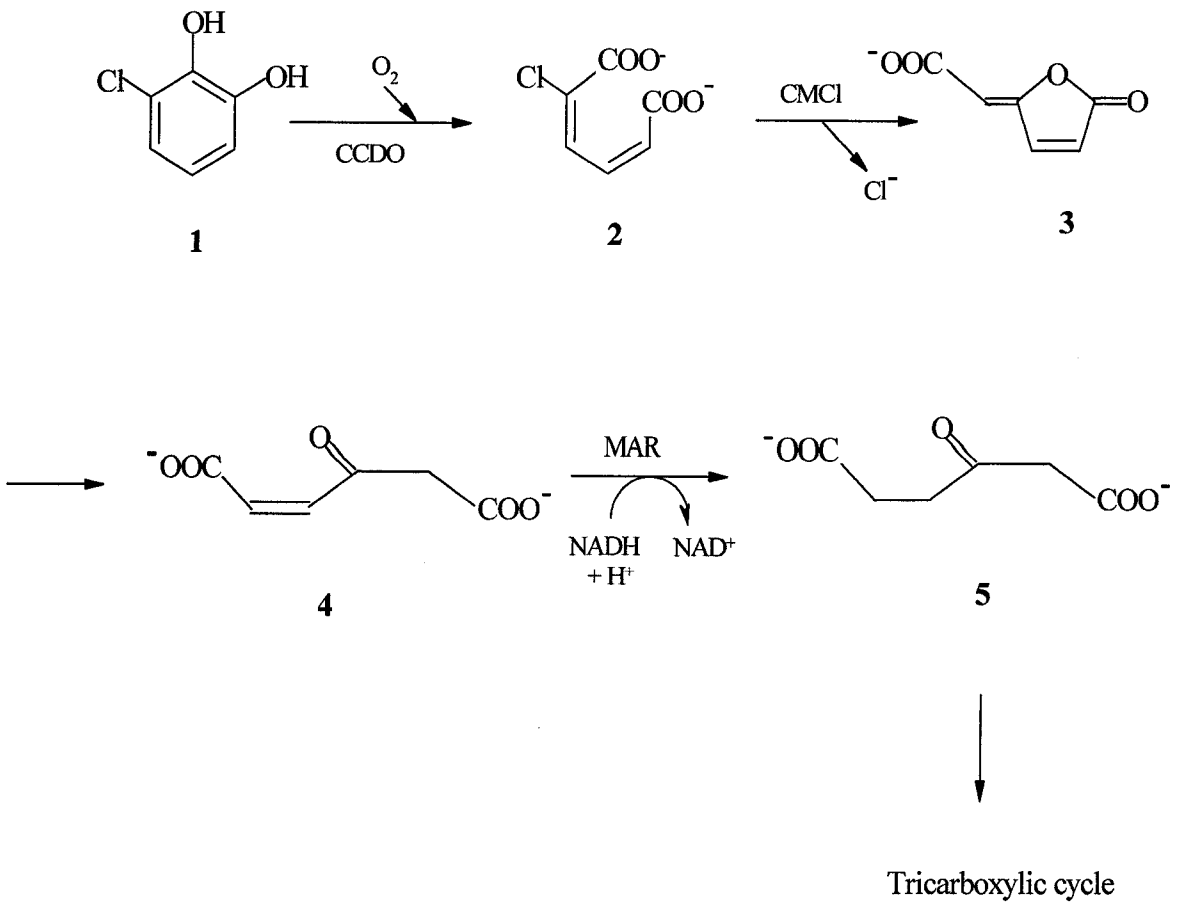


Fig. 1.17. Dehydrodehalogenation of 2-chloro-*cis,cis*-muconate (compound **2**) during the degradation of 3-chlorocatechol through the modified *ortho* pathway (adapted from Fetzner et al., 1998). Compounds: **1**, 3-Chlorocatechol; **2**, 2-chloro-*cis,cis*-muconate; **3**, *trans*-dienelactone; **4**, maleylacetate; **5**, 3-oxoadipate. Enzymes: CCDO, Chlorocatechol 1,2-dioxygenase; CMCI, chloromuconate cycloisomerase; MAR, maleylacetate reductase.

1.3 Polychlorinated Biphenyl

1.3.1 General Description

PCBs, aromatic compounds having two chlorinated benzene rings linked together, were synthesized between the early 1920s and 1978 by a variety of manufacturers in the United States, Europe, and Japan. Commercial mixtures of PCBs were prepared by direct chlorination of biphenyl at temperature above 150 °C in the presence of a catalyst such as iron fillings or ferric chloride. The degree of chlorination of the products is dependent upon the contact time of the chlorine and biphenyl (Robinson and Lenn, 1994). Theoretically there are 209 possible congeners (i.e. isomers) of PCBs (Table 1.1), but as a result of mechanistic and statistical constraints 20 PCBs are absent in commercial preparations (Hutzinger *et al.*, 1974). Typically, a synthetic PCB mixture will contain between 60 and 80 different chlorinated biphenyls (Boyle *et al.*, 1992). By using the IUPAC convention, PCBs are labeled according to ring and position of the substituents (see Fig. 1.18, compound I). The commercial mixtures exist under different trade name as Fenclor, Delor, or Aroclor. Among the commercial mixtures, the Aroclor series were most widely used and are named according to the number of carbon atom in the biphenyl nucleus and the percentage by weight of chlorine (Table 1.2). For example, Aroclor 1242 contains 42 % of chlorine (Robinson and Lenn, 1994).

The commercial mixtures of PCBs differ in physical appearance, with Aroclor 1242-1248 being clear liquids, 1254 – 1262 are light colored oils, and 1268 an off-white powder (Robinson and Lenn, 1994). Aroclor 1242 was the most widely used Aroclor, with a range of chlorobiphenyls containing up to five chlorine atoms. PCBs are highly hydrophobic compounds with water-octanol partition coefficients (log K_{ow}) increasing with increasing

Table 1.1. Number of possible isomers and percent chlorine for different PCBs

PCB	PCB chlorination level	Empirical formula	Nominal molecular weight	Number of possible isomers	Percent chlorine by weight
1-3	monochlorobiphenyls	$C_{12}H_9Cl$	188.5	3	18.8
4-15	dichlorobiphenyls	$C_{12}H_8Cl_2$	223	12	31.8
16-39	trichlorobiphenyls	$C_{12}H_7Cl_3$	257.5	24	41.3
40-81	tetrachlorobiphenyls	$C_{12}H_6Cl_4$	292	42	48.6
82-127	pentachlorobiphenyls	$C_{12}H_5Cl_5$	326.5	46	54.3
128-169	hexachlorobiphenyls	$C_{12}H_4Cl_6$	361	42	58.9
170-193	heptachlorobiphenyl	$C_{12}H_3Cl_7$	395.5	24	62.8
194-205	octachlorobiphenyls	$C_{12}H_2Cl_8$	430	12	66.0
206-208	nonachlorobiphenyls	$C_{12}HCl_9$	464.5	3	68.7
209	decachlorobiphenyls	$C_{12}Cl_{10}$	499	1	71.2

Adapted from Environmental agency, UK (2003).

Table 1.2. Approximate congener composition (as a percentage) of several Aroclor mixtures

	Aroclor mixtures						
	1016	1221	1232	1242	1248	1254	1260
biphenyl		11	6				
monochlorobiphenyls	1	51	26	1			
dichlorobiphenyls	20	32	29	17	1		
trichlorobiphenyls	57	4	24	40	23		
tetrachlorobiphenyls	21	2	14	32	50	11	
pentachlorobiphenyls	1	1	10	10	20	49	12
hexachlorobiphenyls					1	34	46
heptachlorobiphenyl						6	36
octachlorobiphenyls							6
nonachlorobiphenyls							
decachlorobiphenyls							

Adapted from Environmental agency, UK (2003).

molecular weight. Within homologous group of PCBs, log K_{ow} is dependent on the pattern of chlorination, and congeners with less *ortho*-substitution generally have greater log K_{ow} (Yeh and Hong, 2002). The solubility of PCBs in water is dependent on the percentage of chlorine present and the position of the chlorine atoms (Table 1.3) (Robinson and Lenn, 1994). Aroclor 1242 is soluble at 200 ppb, whereas Aroclor 1260 is less soluble at 25 p.p.b. Among the individual congeners, 2-monoCB appears to be the most soluble (Table 1.3). The Henry's law constant of PCB congeners also increases with increasing temperature (Bamford et al., 2000).

The physical and chemical properties of PCBs made them industrially useful. Aroclor mixtures are extremely thermostable to temperature as high as 350 °C and resistant to oxidation, acids, and bases (Boyle et al., 1992). As a result of the excellent thermal and electrical properties of PCBs, a significant amount of the mixtures were used in a wide variety of commercial applications. PCBs were used as dielectric fluids in capacitors and transformers, industrial fluids in hydraulic systems and gas turbines, manufacture of adhesive, textiles and printing (Robinson and Lenn, 1994). Although, it is generally accepted that the widespread commercial applications of PCBs was responsible for its anthropogenic release into the environment, however, non-anthropogenic sources of PCBs have also been identified. Three pentachlorobiphenyl (PtCB) isomers were detected in the volcanic ash from Mount St. Helens in Washington State, USA. The substantial amount of inorganic chloride in the ash provided evidence that pyrolysis of polymeric aromatic compounds from degraded plant and soil organic matter in the presence of high amount of inorganic chloride produced the PtCB isomers (Pereira et al., 1980).

Table 1.3. Predicted solubilities of individual PCB congeners in water

PCB Congener	Solubility (ppm)
2-MonoCB	5.8
3-MonoCB	4.8
4-MonoCB	0.76
2',2-DiCB	1.3
2,3-DiCB	1.4
2,4-DiCB	1.1
2,4'-DiCB	0.64
2,5-DiCB	1.5
2,6-DiCB	2.6
3,3'-DiCB	0.71
3,4-DiCB	0.40
3,5-DiCB	0.65
4,4'-DiCB	0.02

Adapted from Dunnivant et al., 1992

PCBs are highly hydrophobic and it has been estimated that some 10 x 10^7 kg reside in the biosphere as contaminants in air, soil, sediments, rivers and wastes streams, and in lipoidal compartments of plants and animal wildlife (Boyle et al., 1992). PCBs have been detected in marine foodweb and in tree barks and growth rings (Meredith and Hites, 1987; Boris et al., 2005). Elevated concentrations of PCBs have also been reported in soils, plants and marine systems in the Canadian Arctic (Bright et al., 1995a, 1995b), and concentrations exceeding the 50 ppm that is set out under the Canadian Environmental Protection Act has been found in two sites (Environmental Sciences Group, 1996).

Exposure to PCBs is known to cause numerous human health effects including reproductive effects, embryotoxicity, oncogenicity, and estrogenic endocrine disruption as well as probable human carcinogenicity (Danse et al., 1997). Some PCBs are metabolized in human to electrophilic products that could react with DNA to form adducts (Zhao et al., 2004), and certain hydroxylated-PCBs display epigenetic toxicity by interfering with intercellular communication (Satoh et al., 2003).

1.3.2 Aerobic Degradation of PCBs

Aerobic degradation of PCBs is widespread among various species of bacteria and fungi. PCB-degrading bacteria belong predominantly to the genera *Pseudomonas*, *Alcaligenes*, *Achromobacter*, *Arthrobacter*, *Moraxella* and *Rhodococcus* (Furukawa, 1994; Abramowicz, 1990; Bedard and Haberl, 1990). Biodegradation of PCBs have been reported in selected groups of eukaryotic microorganisms that include white-rot fungi, *Aspergillus*, *Candida*, *Cunninghamella* and *Saccharomyces* (Yadav et al., 1995; Sasek et al., 1993; Dmochewitz and Ballschmiter, 1988; Dodge et al., 1979; Tejedor et al., 1979).

Polychlorinated biphenyl-degrading bacterial strains are generally known to utilize biphenyl using the same sets of enzymes (Ahmed and Focht, 1973). It is widely accepted that bacteria able to grow on biphenyl usually have the ability to cometabolize various PCB congeners (Kohler et al., 1988). Degradation of (chloro)biphenyl is usually initiated by biphenyl-2,3-dioxygenase, an enzyme that belong to a large family of Rieske non-heme iron oxygenases (Gibson and Parales, 2000). This group of enzymes consists of a terminal oxygenase that is composed of a large α- and a small β-subunit, a ferredoxin, and a ferredoxin reductase. The ferredoxin and ferredoxin reductase act as an electron transport system that transfer electrons from NAD(P)H to the terminal oxygenase. The product of this reaction, *cis*-2,3-dihydro-2,3-dihydroxybiphenyl, is dehydrogenated by a dehydrogenase to form 2,3-dihydroxybiphenyl that is cleaved by an extradiol 2,3-dihydroxybiphenyl-1,2-dioxygenase to produce 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoate (HOPDA) (Fig. 1.18). The HOPDA compound is hydrolyzed at a carbon-carbon bond by HOPDA-hydrolase to yield benzoate and 2-hydro-2,4-pentadienoate. These series of reactions is referred to as

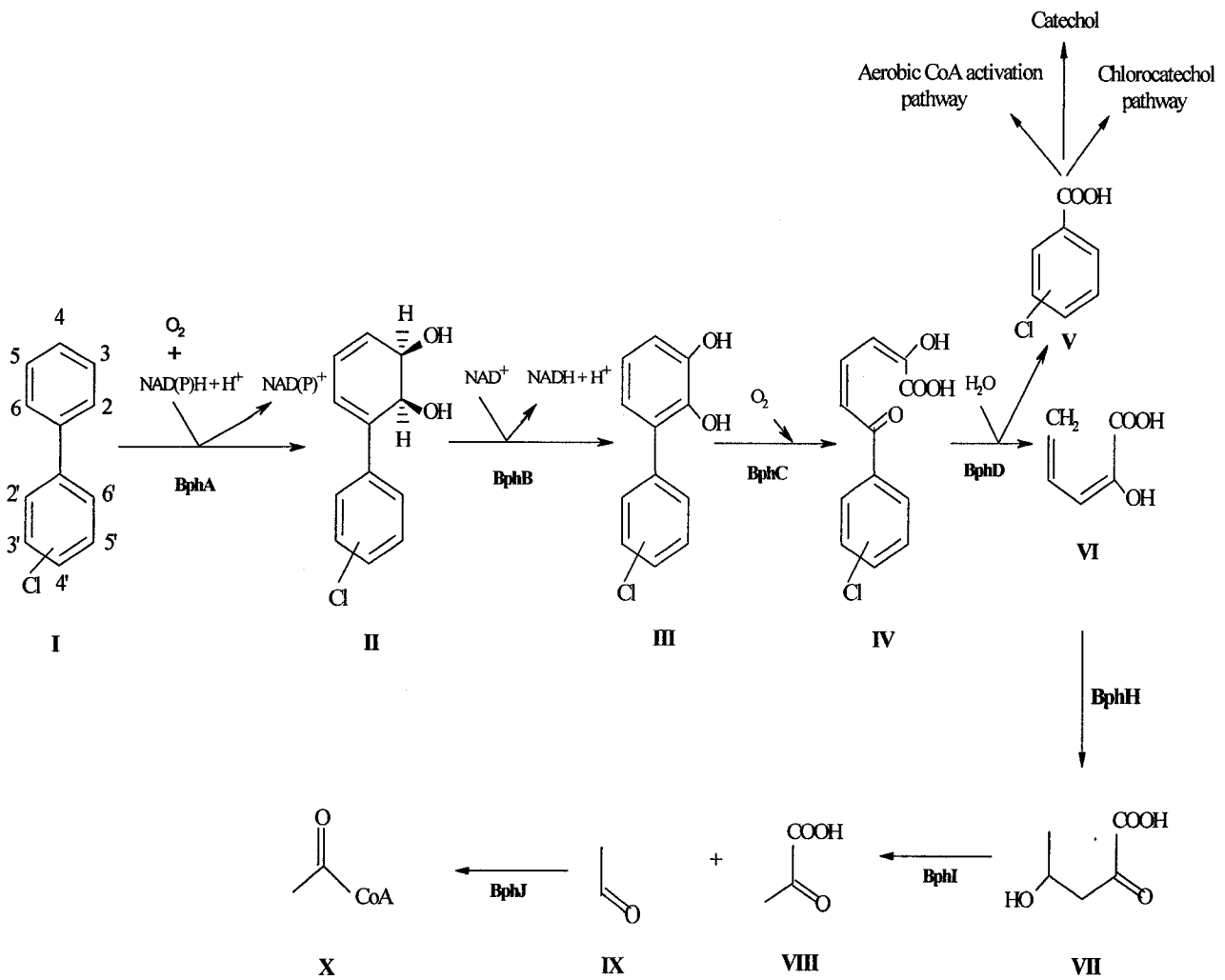


Fig. 1.18. Pathway for bacterial aerobic degradation of (chloro)biphenyl. Compounds: I, (chloro)biphenyl; II, 2,3-dihydroxy-4-phenylhexa-2,4-diene (dihydrodiol compound); III, 2,3-dihydroxybiphenyl; IV, 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoic acid (HOPDA); V, (chloro)benzoic; VI, 2-hydroxypenta-2,4-dienoic acid; VII, 4-hydroxy-2-oxovaleric acid; VIII, pyruvic acid; IX, acetaldehyde; X, acetyl-CoA. Enzyme activities: bphA , biphenyl dioxygenase; bphB, dihydrodiol dehydrogenase; bphC, 2,3-dihydroxybiphenyl dioxygenase; bphD, 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoic acid hydrolase; bphH, (adapted from Furukawa and Arimura, 1987; Kikuchi et al., 1994). Numbering scheme for compound I : 2, and 2', *ortho*-; 6 and 6', *ortho*-; 4 and 4', *para*-; 3 and 3', *meta*-; 5 and 5', *meta*-position.

biphenyl-upper pathway (Pieper, 2005). The benzoate is a dead-end metabolite in certain strains (e.g. *Pseudomonas* sp. NC1B 10643 (Smith and Ratledge, 1989). The benzoate could be metabolized further by benzoate-1,2-dioxygenase followed by dehydrogenation of dihyrodihydroxybenzoate to form catechol as an intermediate. The 2-hydro-2,4-pentadienoate compound could be degraded further to acetyl-CoA (Fig. 1.18).

Many natural occurring bacteria have been reported to grow on one or more of the monochlorinated biphenyl isomers. These organisms may utilize the non-chlorinated ring as a carbon and energy source and accumulate the corresponding chlorobenzoic acid (CBA) (Ahmed and Focht, 1973; Barton and Crawford, 1988; Bevinakatti and Ninnekar, 1993; Masse et al., 1984; Kim and Picardal, 2000). Growth of these strains on chlorobiphenyls is achieved by their ability to utilize the pentadiene compound produced after ring fission of the non-chlorinated ring. The mineralization of dichlorinated biphenyl isomers have also been reported in few naturally occurring bacterial strains. Kim and Picardal (2001) isolated two strains capable of growth on all monoCBs, and 2,2'- or 2,4'-diCB. Ability of strain SK-4 to grow on 2,2'- or 2,4'-diCBs indicates that the bacterium could utilize a chlorinated ring as a carbon and energy source, although the bacterium failed to grow on 2-, 3-, 4-CBA, and other diCBs and triCBs that were tested. Strain SK-3 utilized 2,4'-diCB, but failed to grow on 2,2'-diCB and other diCBs and triCBs that were tested. However, unlike strain SK-4, strain SK-3 did not produce 2- and 4-CBA as intermediates (Kim and Picardal, 2001). It has been established that the inability of most PCB-degrading strains to utilize PCB as a growth substrate is due to the absence of chlorobenzoate-degrading enzymes in these bacteria. Thus, attempts to enhance mineralization of low chlorinated PCB include the use of mixed cultures consisting of PCB-degraders and chlorobenzoate degraders, and the use of hybrid strains

obtained from mating experiments between chlorobenzoate-degrading strains and PCB-degrading strains (Adriaens et al., 1989; Havel and Reineke, 1991; Hickey et al., 1992). A more elegant approach involves the construction of recombinant strains with the ability to utilize chlorinated ring as a carbon and energy source. Hrywna et al. (1999) constructed recombinant variants that are capable of significant growth on 2- or 4-monoCB by cloning plasmid-encoded chlorobenzoate dehalogenase or *ortho*-halobenzoate 1,2-dioxygenase gene into PCB-cometabolizing *Comamonas testosteroni* VP44 (Fig. 1.19).

The transformation of PCBs often results in the accumulation of chlorinated dead-end metabolites, usually CBAs (Furukawa et al., 1979b). Further transformation of CBAs has occasionally been reported (Hrywna et al., 1999; Layton et al., 1992; Sondossi et al., 1992; Bedard and Haberl, 1990). One of the transformation products of CBAs are chlorocatechols. Transformation products of some CBAs may inhibit enzymes of the biphenyl pathway. The *meta*-fission of 3-chlorocatechol that is formed from 2- and 3-monoCB produced a highly reactive acyl halide (5-chloroformyl-2-hydroxypenta-2,4-dienoic acid) which inactivates macromolecules by binding irreversibly onto them (Sondossi et al., 1992). However, the 4-chlorocatechol that is produced from 4-monoCB is usually transformed without restrictions (Fig. 1.20) (Arensdorf and Focht, 1994). Dechlorination of CBAs is initiated in some bacterial strains by a *para*-directed hydrolytic dehalogenase enzyme (Reineke and Knackmuss, 1988) or by *ortho*-directed elimination of chloride by a three component *ortho*-halobenzoate 1,2-dioxygenase (Romanov and Hausinger, 1994). Oxidative elimination of chloride from *ortho*-substituted CBAs is also catalyzed in some strains by a two-component 2-halobenzoate-1,2-dioxygenase (Fetzner et al., 1998).

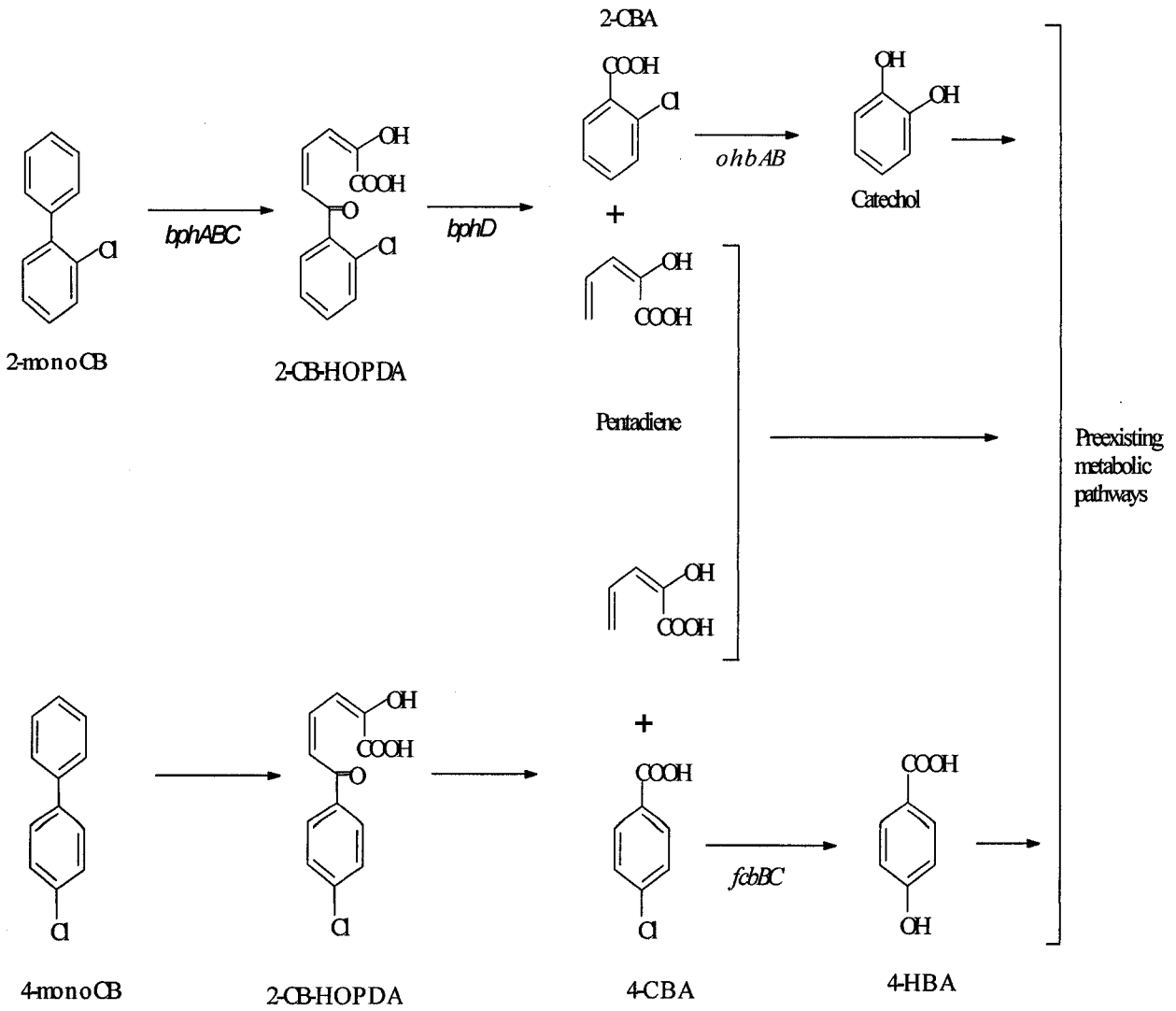


Fig. 1.19. Recombinant pathways for degradation of 2-monoCB (top) and 4-monoCB (bottom), constructed by upgrading pre-existing pathways for oxidation of (chloro)biphenyl, 4-hydroxybenzoate (4-HBA), catechol, and pentadiene with the aromatic ring delahogenase genes *ohbAB* (*ortho*-halobenzoate dehalogenase enzyme) and *fcbABC* (*para*-directed hydrolytic dehalogenase enzyme), respectively (HOPDA: 2-hydroxy-6-oxo-6-(4-chloropheny)hexa-2,4-dienoic acid) (adapted from Hrywna et al., 1999).

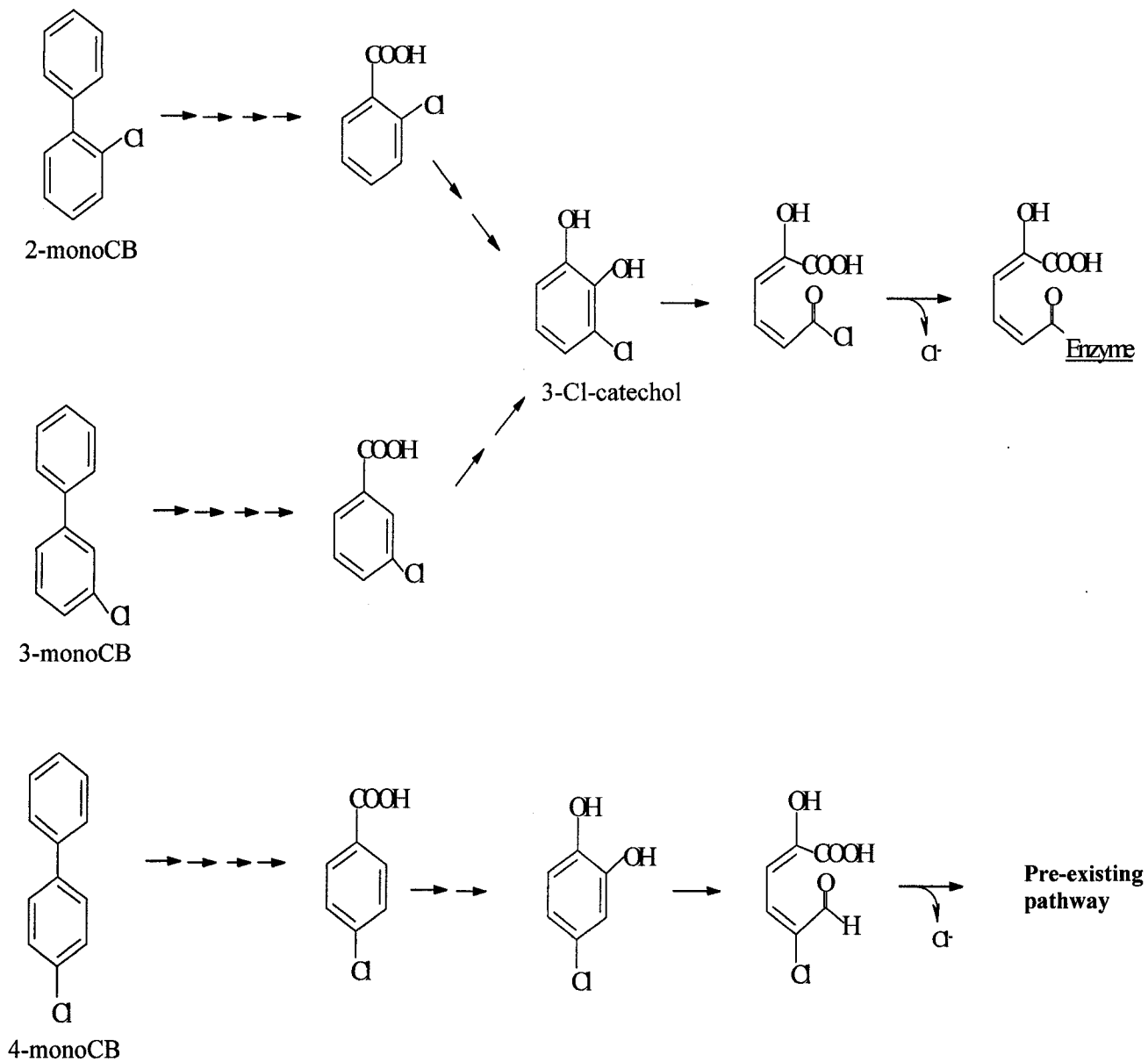


Fig. 1.20. Proposed pathway for metabolism of 2-, 3-, or 4-monoCB by strain P166.

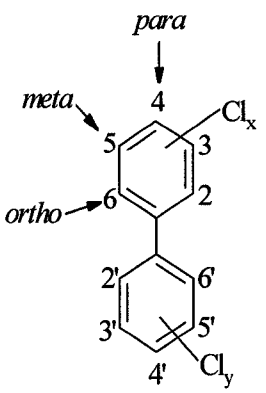
2- and 3-monoCBs are transformed to 3-chlorocatechol (3-Cl-catechol), which is *meta*-cleaved to form a reactive acyl halide, which condenses to cellular macromolecules. 4-MonoCB is transformed to 4-chlorocatechol, which is metabolized via a *meta*-cleavage pathway, with eventual release of chloride (adapted from Arensdorf and Focht, 1994).

Although certain PCBs serve as substrates for biphenyl dioxygenases, PCB-degrading organisms do not usually use PCBs as an energy source, but rather catabolize these substrates cometabolically. To a large extent, the spectrum of PCBs that can be transformed by an organism is determined by the specificity of the dioxygenase enzyme (Pieper, 2005). For example, the oxidation of 2,4,5,2',4',5'-hexaCB and congeners with similar pattern of chlorination by cells of *Alcaligenes eutrophus* H850 is achieved by biphenyl-3,4-dioxygenase (Bedard et al., 1987a). The possession of biphenyl-3,4-dioxygenase permits the hydroxylation of highly chlorinated PCBs. There have been considerable efforts to increase the substrate range of biphenyl dioxygenases by directed mutagenesis and directed evolution (Barriault *et al.*, 2002; Suenaga et al., 2002). However, studies indicates that complete degradation of PCBs could only be effected with recombinant strains containing metabolic pathways not only specifically engineered for expanding the initial dioxygenase, because other enzymes downstream of the pathway have been shown to be less tolerant of chlorinated intermediates. Thus, in many cases, metabolites of the upper-pathway are formed as dead-end products (Seah *et al.*, 2000; Bruhlmann and Chen, 1999; Seeger et al., 1997; Bedard and Haberl, 1990).

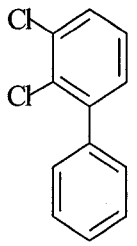
In several cases, biphenyl is required as a carbon source and inducer of the enzymes required for the degradation of PCBs (Maltseva et al., 1999; Bedard and Haberl, 1990; Ahmed and Focht, 1973). Biphenyl-degrading enzymes are also produced in the cells of some bacterial strains grown on glycerol, glucose, or plant compounds carvone, limonene, isoprene, and *p*-cymene (Billingsley et al., 1999; Billingsley et al., 1997; Gilbert and Crowley, 1997). It is very likely that different sets of enzymes are induced after growth on different carbon sources. *Pseudomonas* strain LB400 grown on biphenyl degraded 2-CBA

and 4-CBA, but cells grown on glycerol did not degrade these compounds (Billingsley et al., 1997). When biphenyl, glycerol, or glucose grown cells of strain LB 400 were incubated with 2,2'-diCB or 2,5,4'-triCB, the rates and extents of transformation of the compounds and the production of metabolites were higher with biphenyl-grown cells (Billingsley et al., 1999).

The structural class of PCBs and the likelihood that a PCB congener will be degraded depend on the degree and pattern of chlorination of the congener (Fig. 1.21). Previous studies have established that the: degradability of PCBs is strain dependent; congeners with double *para*-substituents (i.e. 4,4'-), and that congeners with double *ortho*-substituents on a single ring or both rings could be resistant to degradation; congeners with unchlorinated ring are more susceptible to degradation than congeners having chlorine on both rings; congeners with fewer chlorine substituents are more susceptible to degradation than congeners with higher numbers of chlorine; oxidation of PCBs in most PCB-degrading bacteria is initiated by biphenyl-2,3-dioxygenase at the 2,3- or 5,6- positions of the chlorophenyl rings; oxidation of PCBs at the 3,4- or 4,5- positions by biphenyl-3,4-dioxygenase is rare in PCB-degrading bacteria; the types of products that are formed from PCBs are largely strain-independent and are determined by the chlorine substitution pattern on the reacting ring; oxidation of unsubstituted ring or a 2-chlorophenyl ring would result in high yield of chlorobenzoic acids, oxidation of a 3-chlorophenyl ring would result in moderate yield of chlorobenzoic acids, and oxidation of a 4- or 2,4-chlorophenyl ring would result in low yield of chlorobenzoic acids and apparent accumulation of *meta* ring-fission product (Bedard and Haberl, 1990; Bedard *et al.*, 1987a, 1987b; Bedard *et al.*, 1986).

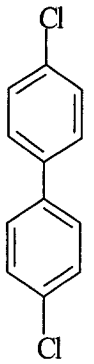


2,3-Dichlorobiphenyl



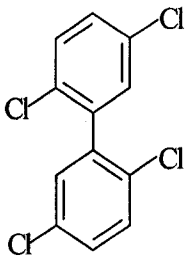
Single Ring Substitution

4,4'-Dichlorobiphenyl



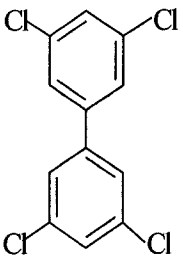
Double *Para* Substitution
No Free 3,4 or 4,5 Sites

2,5,2',5'-Dichlorobiphenyl



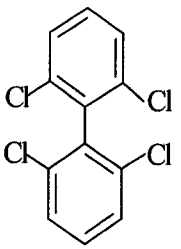
No Free 2,3 or 5,6 Sites

3,5,3',5'-Dichlorobiphenyl



No Adjacent
Unchlorinated Sites

2,6,2',6'-Dichlorobiphenyl



Two or More *Ortho*
Chlorines. Steric Hindrace

Fig. 1.21. Structural classes of PCB congeners showing numbering system and examples of congeners that represent different structural challenges for PCB-degradative enzymes (adapted from Bedard *et al.*, 1986).

1.3.3 Anaerobic Degradation of PCBs

To date the most promising option for biological treatment of PCB-contaminated sites is a sequential anaerobic-aerobic scheme where highly chlorinated congeners are anaerobically dechlorinated to produce lightly chlorinated congeners that can be degraded aerobically (Quensen et al., 1988). Highly chlorinated congeners in commercial mixtures, such as Aroclor 1242, 1248, 1254, and 1260, can be reductively dechlorinated by microorganisms. Reductive dechlorination generally reduces the chlorine content of PCB mixtures and the major products include monoCBs and diCBs (Mohn and Tiedje, 1992).

Studies have demonstrated that reductive dechlorination of PCBs is a relevant route for the transformation of PCBs in the environment. There is evidence that PCBs are reductively dechlorinated in lake and river sediments (Magar *et al.*, 2005; Imamoglu *et al.*, 2004; Alder *et al.*, 1993). Reductive dechlorination of PCBs has also been demonstrated in laboratory studies (Master *et al.*, 2002; Drenzek *et al.*, 2001). Previous attempts to isolate and identify the dehalogenating microorganisms in PCB-dechlorinating enrichment cultures were not successful (Mohn and Tiedje, 1992). Thereafter, a defined 2,3,5,6-tetraCB dechlorinating enrichment culture was shown to include strains belonging to the epsilon subgroup of the class Proteobacteria, the low-G+C gram-positive subgroup, the *Thermotogales* subgroup, and a strain with sequence similarity to the deeply branching species *Dehalococcoides ethenogenes* (Holloman *et al.*, 1998). More recently, however, two bacterial strains, designated as OUT 1 and *o*-17, were identified by selective enrichment and by denaturing gradient gel electrophoresis as the PCB-dechlorinating strains in PCB-dechlorinating cultures (Wu *et al.*, 2002; Cutter *et al.*, 2001).

Microbial dechlorination of PCBs is a specific process. The pattern of dechlorination of PCBs is determined by the source of the anaerobic enrichment sediments (Fig. 1.22). Anaerobic dechlorination processes are termed according to the site of dechlorination of PCBs. *Meta* and *para* dechlorination are termed processes M and Q, respectively. A combination of *meta* and *para* dechlorination is referred to as process C (Brown et al., 1987a, 1987b). Though less frequently observed, the removal of *ortho*-chlorine from PCBs has been reported (van Dort and Bedard, 1991). The removal of *meta* and/or *para* chlorines in PCBs results in accumulation of *ortho* plus *para*- and/or *ortho*-substituted congeners as the by-products. Depending on the level of chlorination of the target PCBs, the products of anaerobic dechlorination of PCBs may include *ortho*-, *para*-, or *ortho*- plus *para*-substituted monoCBs, diCBs, triCBs, or tetraCBs (Quensen and Tiedje, 1998; Bedard and Quensen, 1995). Congeners including 2-, 4-, 2,4-, 2,6-, 2,2'-, 2,4'-, 2,2',4, and 2,4,4'-chlorobiphenyl comprises 70 to 85 molar % of the products of anaerobic dechlorination of Aroclor 1242 (Quensen *et al.*, 1988). The lightly chlorinated products can then be treated aerobically leading to complete mineralization of the congeners (Quensen *et al.*, 1988).

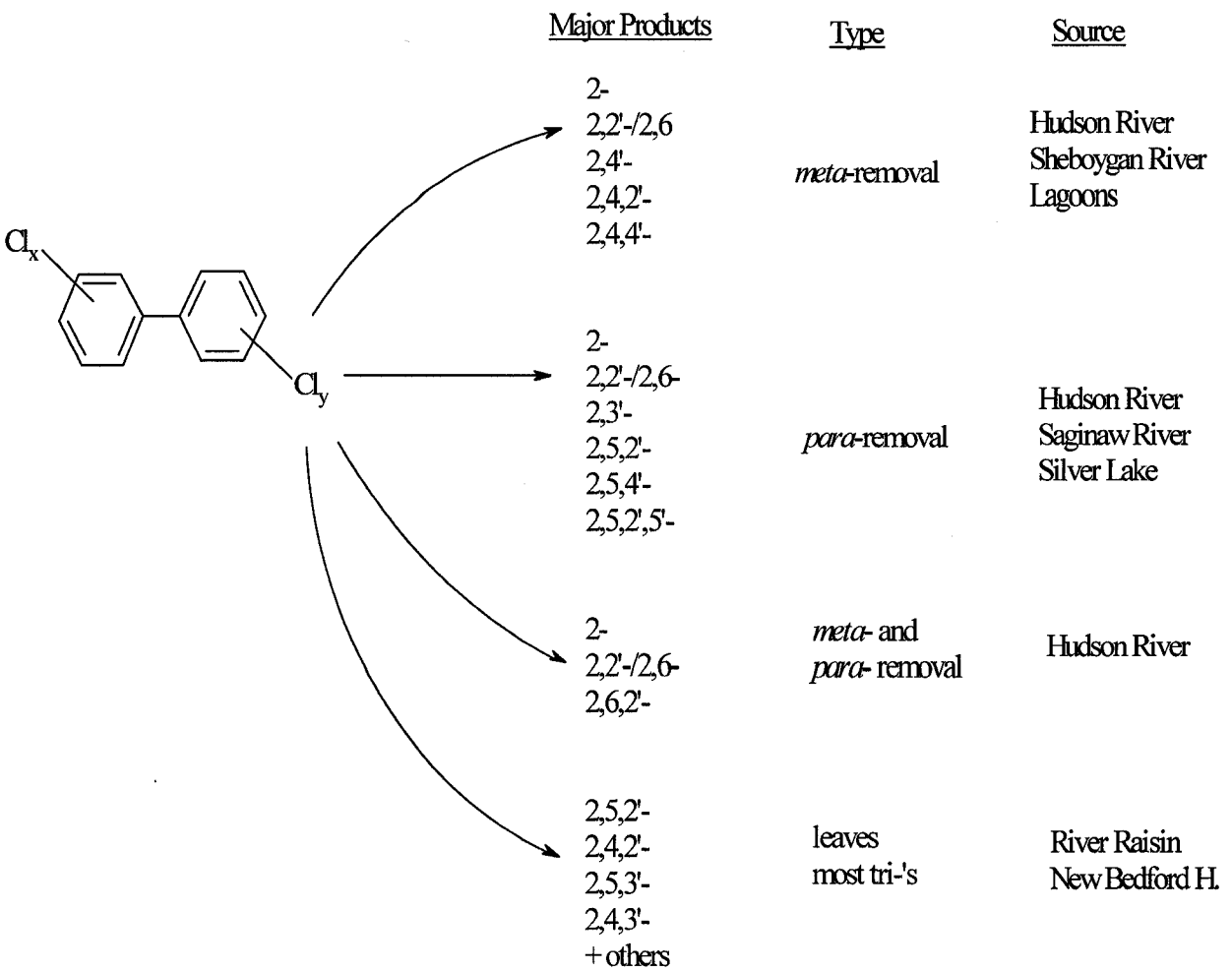


Fig. 1.22. Different pattern of PCB dehalogenation depending on inoculum source. (adapted from Mohn and Tiedje, 1992).

1.4. 2,4'-Dichlorobiphenyl: General Description and Aerobic Degradation

As a model *ortho*-(2) plus *para*-(4') substituted chlorinated biphenyl compound, 2,4'-diCB contains two phenyl rings and two chlorine atoms. One of the phenyl rings is substituted at the *ortho* (i.e. 2) position and the other ring is substituted at the *para* (i.e. 4) position (see Fig. 1.21.) The compound is present at relatively abundant concentrations in some commercial mixtures of PCBs that include Aroclor 1016, 1221, 1232, and 1242 (Table 1.2). It is present at low concentrations in Aroclor 1248, and at trace levels in Aroclor 1254 and 1260 (Frame et al., 1996). 2,4'-diCB is also a by-product of anaerobic dechlorination of highly chlorinated PCB mixtures (Quensen and Tiedje, 1998; Zwiernik *et al.*, 1998; Nies and Vogel, 1990), and it has been detected in samples of sediments, that were previously contaminated with mixtures of highly chlorinated PCBs, as a possible by-product of anaerobic dechlorination (Flanagan and May, 1993). The compound was proposed as a model chlorinated biphenyl compound that can be used to study bacteria having the ability to degrade *ortho*- plus *para*-substituted congeners more efficiently, because this group of bacteria could be used in the aerobic stage of the two-phase anaerobic-aerobic PCB biotreatment process (Maltseva *et al.*, 1999).

Biodegradation of pure 2,4'-diCB by several bacterial strains has been reported before (Rybkina *et al.*, 2003; Maltseva *et al.*, 1999; Bedard and Haberl, 1990; Furukawa *et al.*, 1979a). Microbial degradation of this congener in mixtures of PCBs is also well documented (Master and Mohn, 1998; Mohn *et al.*, 1997; Bedard *et al.*, 1987b; Bedard *et al.*, 1986). Few bacterial strains have been shown to utilize 2,4'-diCB as a source of carbon and energy (Rybkina *et al.*, 2003; Kim and Picardal, 2001). A coculture of strain LB400 and *Pseudomonas putida* mt-2a completely mineralized 2,4'-diCB and formed 4-chlorocatechol

as an intermediate (Fig. 1.23) (Potrawfke *et al.*, 1998). 2,4'-DiCB is cometabolized by most PCB-degrading bacteria (Maltseva *et al.*, 1999; Fava *et al.*, 1994; Bedard and Haberl, 1990). Bacterial strains that were shown to cometabolize 2,4'-diCB include members of the genus *Acinetobacter*, *Alcaligenes*, *Burkholderia*, *Comamonas*, *Corynebacterium*, *Microbacterium*, *Pseudomonas*, and *Rhodococcus*.

Bacterial oxidation of 2,4'-diCB is initiated on one of the substituted rings or on both rings (Bedard and Haberl, 1990). Oxidation of the 2-chlorophenyl ring at the 2,3- position results in the elimination of chloride, and the formation of 2,3-dihydroxy-4-chlorobiphenyl which undergoes a *meta* fission that is catalyzed by 2,3-dihydroxybiphenyl dioxygenase to generate 2-hydroxy-6-oxo-6-(4'-chlorophenyl)-hex-2,4-dienoic acid (monochloro-HOPDA). The HOPDA compound is then converted to 4-CBA and a pentadiene compound by HOPDA-hydrolase (Fig. 1.23) (Potrawfke et al., 1998). Bacterial strains that are capable of oxidizing both 2- and 4-chlorophenyl rings likely transformed the compound through analogous pathways to produce 4-CBA in addition to 2-CBA that is generated as an end-product of an alternate pathway that include 2,3-dihydroxy-2,3-dihydro-2,4'-dichlorobiphenyl, 2,4'-dichloro-2,3-dihydroxybiphenyl, and 3-chloro-2-hydroxy-6-oxo-(2-chlorophenyl)hexa-2,4-dienoic acid as intermediates (Maltseva *et al.*, 1999; Furukawa *et al.*, 1979a). Studies on the regiospecificity of 2,4'-diCB-degrading strains suggests that predominant oxidation of 2-chlorophenyl ring lead to the formation of 4-CBA as a major end-product, whereas strains that preferentially oxidized the 4-chlorophenyl ring accumulated 3-chloro-2-hydroxy-6-oxo-(2'-chlorophenyl)hexa-2,4-dienoic acid (dichloro-HOPDA) as a major intermediate with relatively low production of 2- and 4-CBA or 2-CBA

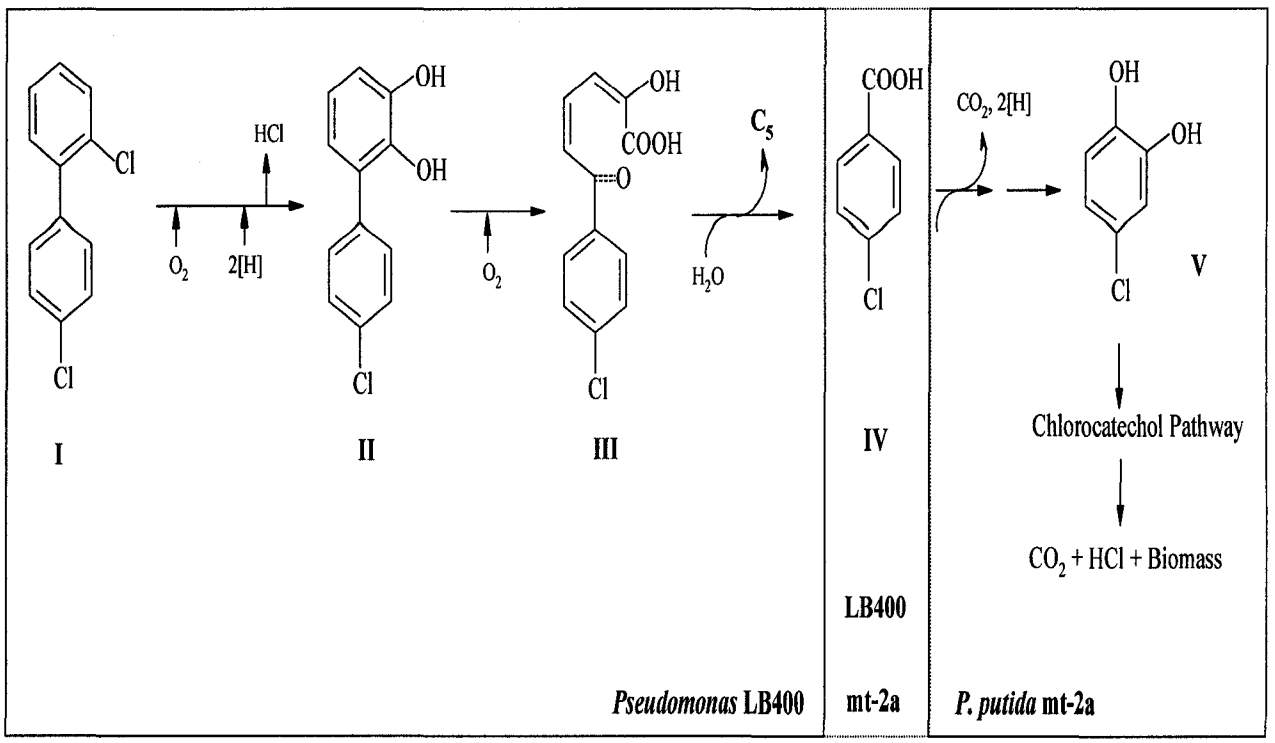


Fig. 1.23. Proposed pathway for the degradation of 2,4'-diCB by the two-species consortium consisting of strain LB400 and strain *P. putida* mt-2a. In *dotted box*, the excretion of 4-chlorobenzoate, a dead-end product of strain LB400, is shown. The 4-chlorobenzoate is taken up and mineralized by strain mt-2a, which had obtained the regulated chlorocatechol pathway genes from strain LB400. Compounds: **I**, 2,4'-dichlorobiphenyl; **II**, 2,3-dihydroxy-4-chlorobiphenyl; **III**, 2-hydroxy-6-oxo-6-(4'-chlorophenyl)-hex-2,4-dienoic acid (mono-chloro-HOPDA); **IV**, 4-chlorobenzoate; **V**, 4-chlorocatechol; C_5 indicates pentadiene compound (adapted from Potrawfke *et al.*, 1998).

only (Maltseva *et al.*, 1999; Bedard and Haberl, 1990). The non-enzymatic products of bacterial metabolism of 2,4′-diCB includes 2-chloroacetophenone and two isomers of 2,4′-dichloro-hydroxybiphenyl (Bedard and Haberl, 1990; Furukawa et al., 1979a). Studies conducted by Ahmad *et al.* (1991) using *Pseudomonas testosteroni* B-356 and *P. putida* clone pDA261 suggests that the oxidation of 2- and 4-chlorophenyl rings of 2,4'-diCB could result in multiple conversion pathways (Fig 1.24). In this case, 2,3-dihydroxy-2,4′-dichlorobiphenyl, 2,3-dihydroxy-2′-chlorobiphenyl, and 2- and 4-CBA were formed from 2,4′-diCB. It was suggested that dehalogenation of 4-chlorophenyl ring of 2,4′-diCB caused the formation of 2,3-dihydroxy-2′-chlorobiphenyl.

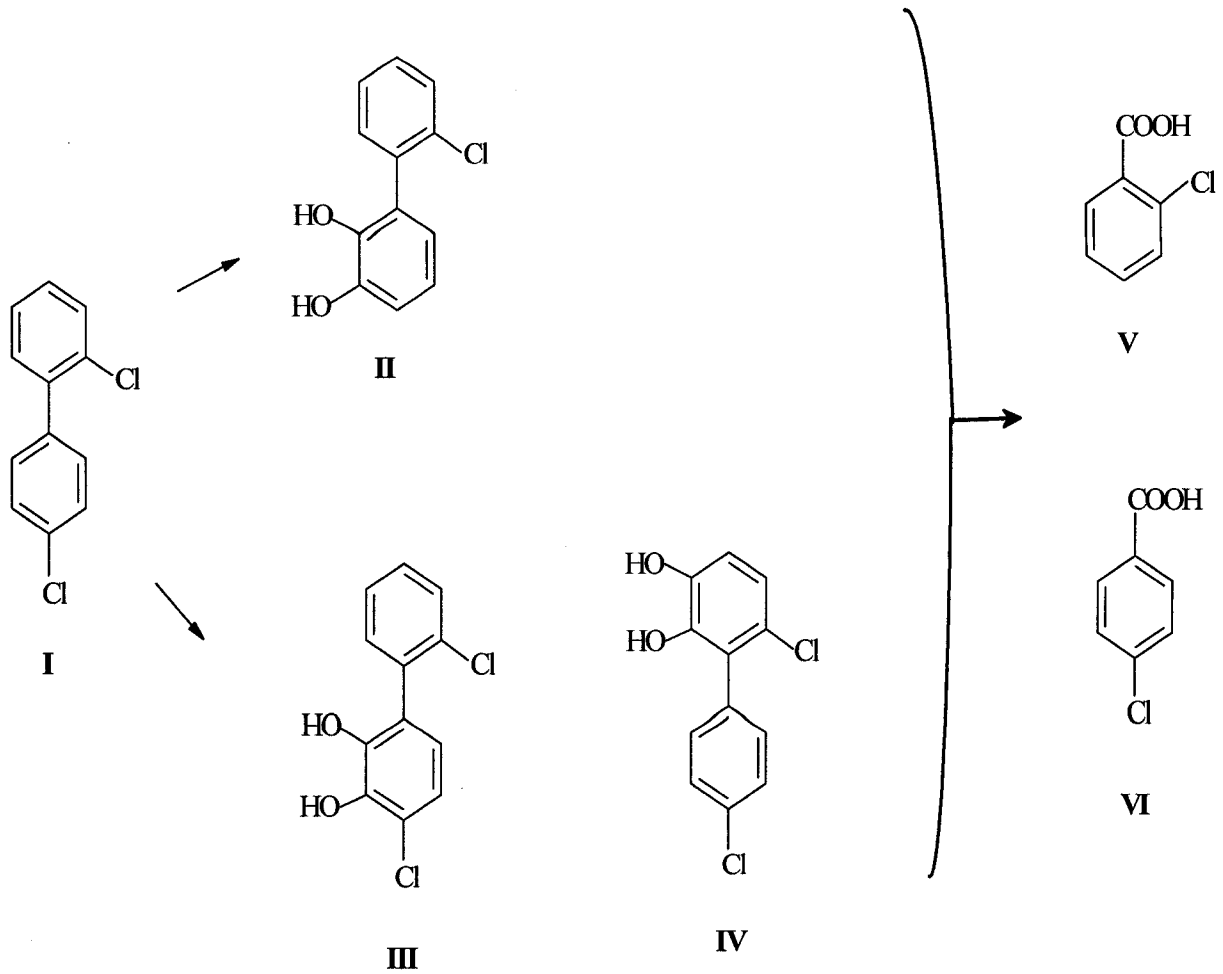


Fig. 1.24. Proposed patterns of conversion of 2,4'-diCB by clones carrying the *bphA* gene from *P. testosteroni* B-356. Compounds: **I**, 2,4'-dichlorobiphenyl; **II**, 2,3-dihydroxy-2'-chlorobiphenyl; **III**, 2,3-dihydro-2,4'-dichlorobiphenyl; **IV**, 5,6-dihydro-2,4'-dichlorobiphenyl; **V**, 2-chlorobenzoate; **VI**, 4-chlorobenzoate (modified from Sondossi *et al.*, 1991).

1.5 Microbial Adaptation to Low Temperature

The life processes of all organisms are adapted for optimum functioning in their respective 'normal' physiological environments. Any change in the environmental conditions is experienced as a stress that threatens the normal metabolic balance and produces a response designed to counter the effects of the disturbing influence. Of all the natural stress conditions that threaten life on this planet, cold is by far the most widespread. A substantial proportion of the ecosphere is subject to subzero temperatures either permanently or seasonally (Franks *et al.*, 1990). Microorganisms are similar to higher organisms in that each species can usually grow only over a temperature span of some 30 ° - 40 °C. As a group, microorganisms can grow at temperatures ranging from sub-zero to boiling point and, although they represent a thermal continuum, individual species are classified as psychrophiles, mesophiles or thermophiles according to their growth temperature range. The cardinal (lower, optimum and upper) growth temperature is used to define two groups of microorganisms, namely psychrophiles and psychrotrophs, which are capable of growing at or close to zero (Russell *et al.*, 1990). Morita (1975) proposed that psychrophiles are organisms having optimum growth temperatures less than 15 °C and upper limits below 20 °C, whereas psychrotrophs (which are still capable of growing at or close to zero) have optimum growth temperatures above 15 °C and upper limits as high as 40 °C in a few cases.

Temperature influences growth rates by affecting the conformation of cellular macromolecules and other constituents, which determine the rates of enzyme reactions. The relation between temperature and reaction rate (k) is describes by the Arrhenius equation: $k = Ae^{-Ea/RT}$, where E_a, activation energy; A, a constant that is related to steric

factors and collision frequency; R, universal gas constant; T, absolute temperature (K). The activation energies for most enzymes are usually of the order of 420 kJ mol^{-1}, therefore a drop in temperature from 20 °C to zero will produce an approximate fourfold decrease in enzyme rate constant (Russell *et al.*, 1990). Most biological systems, including the single biochemical reaction, display a reaction rate 2 to 3 times lower when the temperature is decreased by 10 °C (Q_{10} = 2 to 3). As a result, the activity of mesophilic enzyme is between 16 and 80 times lower when the reaction temperature is shifted from 37 °C to 0 °C (Feller and Gerday, 1997). In contrast, the enzymes of cold-adapted strains are characterized by their high specific activity or turnover number k_{cat}. It is thought that improving k_{cat} offsets the effect of low temperatures on catalytic rate and it thus provides adequate raw metabolic activity to the growing organism. A correlation between conformational flexibility and enzyme activity is the most widely accepted hypothesis accounting for the dominant adaptive traits of psychrophilic enzymes, i.e. their high activity and their weak stability (Feller and Gerday, 1997).

A comparison of the fatty acid composition of two thermophilic, a mesophilic and a psychrophilic species of clostridia indicates that the proportion of unsaturated fatty acid increased in the following order: thermophiles (average, 10%), mesophile (37%), psychrophile (52%) and the psychrophile also had a higher proportion of short-chain fatty acids (Chan *et al.*, 1971). These changes would lower the gel-liquid-crystalline phase transition temperature (T_m) of the lipids (Russell *et al.*, 1990). The observed changes in fatty acid composition of cold-adapted strains are the same as some of those in mesophiles and thermophiles, that is, decrease in growth temperature results in an increase in fatty acid unsaturation or chain shortening (Russell 1984). Alteration of

membrane fluidity is a means of regulating the activity of vital membrane-bound enzymes and transport systems (Russell 1984). These factors and few others have improved the adaptability of cold-adapted microorgansisms to their respective environments, thus enabling them to carry out life processes at rates that are often comparable to those of their mesophilic counterparts.

1.6 Biodegradation of Aromatic Compounds by Cold-Adapted Microorganisms

Biodegradation of aromatic compounds at low temperatures by cold-adapted microorganisms has been reported in several studies. Biodegradation of benzene, toluene, ethylbenzene, xylenes has been observed at low temperatures under aerobic and anoxic conditions (Baraniecki *et al.*, 2002; Aislabie *et al.*, 2000; Braddock and McCarthy, 1996; Bradley and Chapelle, 1995). Aromatic compounds that were transformed or utilized for growth by cold-adapted bacteria includes naphthalene, methylnaphthalenes, 2-ethylnaphthalene, phenanthrene, fluorene, fluoranthene, pyrene, chrysene, 2-methylanthracene, biphenyl, catechol, or phenol (Erickson *et al.*, 2003; Baraniecki *et al.*, 2002; Kato *et al.*, 2001; Aislabie *et al.*, 2000; Dyksterhouse *et al.*, 1995; Siron *et al.*, 1995). Chlorinated aromatic compounds that were observed to be degraded by cold-adapted microorganisms include chlorophenols and PCBs (Tiirola *et al.*, 2005; Master and Mohn, 1998; Jarvinen *et al.*, 1994; Mohn *et al.*, 1997). In addition, catabolic pathways responsible for the degradation of certain aromatic and polyaromatic hydrocarbons are prevalent in cold regions (Margesin *et al.*, 2003; Whyte *et al.*, 1997; Sotosky and Atlas, 1994).

Cold-adapted microorganisms that degrade aromatic hydrocarbons, PAHs, and chlorinated aromatic hydrocarbons include a diverse group of bacteria belonging to the class β-proteobacteria, γ-proteobacteria, α-proteobacteria, Actinobacteria, and gram-positive bacteria with high G + C content. Studies have shown that strains belonging to the genus *Pseudomonas* are predominantly involved in the degradation of aromatic compounds in cold-climates (Ma *et al.*, 2006). In addition, bacterial strains that degrade aromatic compounds at low temperature were also shown to belong to the genus *Acidovorax*, *Arthrobacter, Bordetella*, *Cycloclasticus, Herbaspirillum, Nocardiodes, Novosphingobium, Rahnella,*

Shewanella, *Sphinghomonas*, *Variovorax*, (Ma *et al.*, 2006; Tiirola *et al.*, 2005; Erickson *et al.*, 2003; Kato *et al.*, 2001; Mannisto *et al.*, 2001; Aislabie *et al.* 2000; Dyksterhouse *et al.*, 1995). Reports on degradation of aromatic compounds at low temperature by eukaryotic microorganisms are rare. In contrast to cold-adapted aromatic compounds-degrading microorganisms, a more diverse group of microorganisms have been shown to degrade aromatic compounds at mesophilic temperatures (Atlas, 1981). When compared to mesophilic strains, the isolation and characterization of cold-adapted aromatic compounds-degrading microorganisms is relatively more recent. In addition, there have been relatively few attempts to characterize the microorganisms involved in the degradation of aromatic compounds at low temperature (Williams and May, 1997). In certain cases, studies on the degradation of aromatic compounds at low temperature that focused on microbial degradation of mixtures of compounds or the non-aromatic components contained in the mixtures did not indicate that the aromatic fractions were not degraded. The identity of the microorganisms involved is often unknown (Margesin *et al.*, 2003; Margesin and Schinner, 1998a, 1998b). It is very likely that a more diverse group of microorganisms are involved in the degradation of aromatic pollutants in cold climates than it is presently known, and therefore, it may be too early to conclude that the requirements for cold-adaptation limits the diversity of microorganisms that are involved in the degradation of aromatic compounds in cold climates.

1.7 Biodegradation of Aromatic Compounds by the Genus *Hydrogenophaga*

The genus *Hydrogenophaga* includes seven species of bacteria that are chemoorganotrophic or chemolithoautotrophic (Kampfer *et al.*, 2005). Some species belonging in the genus *Hydrogenophaga* were previously classified in the genus *Pseudomonas* (Bergey, 1994), however, Willems *et al.* (1989) reclassified these species into a separate genus, and they include *Hydrogenophaga flava* (formerly *Pseudomonas flava*), *Hydrogenophaga palleronii* (formerly *Pseudomonas palleronii*), *Hydrogenophaga pseudoflava* (formerly *Pseudomonas pseudoflava* and *Pseudomonas carboxydoflava*) and *Hydrogenophaga taeniospiralis* (formerly *Pseudomonas taeniospiralis*). Recent studies described three new strains, *Hydrogenophaga defluvii*, *Hydrogenophaga atypical*, and *Hydrogenophaga intermedia* (Kampfer *et al.*, 2005; Contzen *et al.*, 2000).

Unlike *Pseudomonas* strains, biodegradation of aromatic compounds by members of the genus *Hydrogenophaga* has not been widely reported. Nonetheless, aromatic compounds that were shown to be degraded by some *Hydrogenophaga* species include dinitrotoluene, 4-aminobenzenesulfonic, 4-carboxy-4'-sulfoazobenzene, 3-hydroxybenzoate, and 4-hydroxy-benzoate (Kampfer *et al.*, 2005; Blumel *et al.*, 1998; Feigel and Knackmuss, 1993). A previous study identified a PCB-degrading bacterial strain as *H. pseudoflava* but the bacterium was not characterized further for its ability to degrade PCB congeners (Joshi and Walia, 1995). There are no reports on the degradation of aromatic compounds by cold-adapted strains belonging to the genus *Hydrogenophaga.*

The isolation of strains that are affiliated to the genus *Hydrogenophaga* from aromatic pollutant-impacted environments suggests that some members of this genus are likely involved in the biodegradation of aromatic compounds in the environments. Several bacterial

clones obtained from a biofilm community of a polluted river were shown to be affiliated to bacterial species that include *H. palleronii* and *H. taeniospiralis* (Brummer et al., 2003). Two waste water isolates, *H. atypical* and *H. defluvii* were shown to degrade 3- and 4-hydroxybenzoate (Kampfer *et al.*, 2005).

Alhough, reports on the degradation of aromatic compounds by strains belonging to the genus *Hydrogenophaga* are relatively rare when compared to well characterized aromatic compounds-degrading strains belonging in the genus *Pseudomonas*, *Rhodococcus*, *Acinetobacter*, or *Alcaligenes*. However, strains belonging to the genus *Hydrogenophaga* have been shown to remove different classes of pollutants including arsenite and fuel oxygenate methyl *tert*-butyl ether (vanden Hoven and Santini, 2004; Hatzinger *et al.*, 2001). In addition, cold-adapted *Hydrogenophaga* species have been isolated, or shown to be present in samples, from permanently or seasonally cold environments (Brummer *et al.*, 2003; Rutter and Nedwell, 1994), therefore, cold-adapted *Hydrogenophaga* strains having the ability to degrade aromatic compounds could be used for bioremediating pollutants in cold climates.

1.8 Research Objectives

The widespread distribution of xenobiotic compounds in the environment, and the presence of these compounds in cold climates have increased interest in cost-effective decontamination technologies. Elevated concentrations of PCBs have been reported in soils, plants and marine systems in the Canadian Arctic (Bright *et al.*, 1995a, 1995b), and PCBs exceeding a concentration of 50 ppm that is set out under the Canadian Environmental Protection Act has been found in two sites (Environmental Sciences Group, 1996). The remote location of these sites makes conventional cleanup technologies very expensive and bioremediation has been proposed as a cost saving treatment option. Successful application of bioremediation for decontamination of these sites would require the use of cold-adapted microorganisms. However, very few studies have described cold-adapted microbial strains having the ability to degrade PCBs. To date, only two studies investigated the degradation of PCBs by cold-adapted bacterial strains and the products of transformation of PCBs by cold-adapted strains are yet to be characterized. In addition, cold-adapted strains that degraded PCBs were closely related to the genus *Pseudomonas* (Master and Mohn, 1998). Therefore, more studies are required to isolate and characterize new PCB-degrading cold-adapted bacterial strains, especially if the strains are species of genera that are poorly characterized for their ability to degrade aromatic compounds.

So far, studies have shown that the most promising biological method for decontamination of PCB-contaminated sites is a sequential anaerobic-aerobic scheme where highly chlorinated PCBs are reductively dechlorinated to produce lightly chlorinated congeners that can be degraded aerobically. The major by-products of anaerobic dechlorination of PCBs are *ortho*-, and *ortho*- plus *para*-substituted congeners (Mohn and

Tiedje, 1992; Quensen *et al.*, 1988). Unfortunately, a setback of the anaerobic-aerobic treatment scheme is that most aerobic PCB-degrading bacteria inefficiently convert *ortho*- plus *para*-substituted congeners to toxic end-products, especially the problematic *meta*-cleavage products (HOPDAs). However, there has been no attempt to investigate the likelihood that the accumulation of such toxic products could be avoided under specific conditions. A previous study suggests that multiple pathways may be used for transformation of PCBs by certain bacterial strains (Ahmad *et al.*, 1991). Therefore, I am proposing that a better control of PCB transformation by strains having multiple conversion pathways could result in more efficient transformation of *ortho*- plus *para*-substituted congeners without excessive accumulation of HOPDA.

In the light of the above, the objectives of the present research were: (i) to isolate and characterize a cold-adapted biphenyl-utilizing strain having the ability to degrade chlorinated biphenyls, and to identify the strain to the genus level; (ii) to determine the ability of the strain to degrade mixtures of low to highly chlorinated PCBs at low temperature and to confirm the pattern of PCBs that are degraded by the strains at low and mesophilic temperatures; (iii) to confirm the existence of multiple pathways for transformation of model *ortho*- plus *para*-substituted congener 2,4'-diCB, and to determine if temperature could be used to regulate the pattern of transformation of 2,4'-diCB in order to prevent excess accumulation of HOPDA and to enhance the recovery of CBAs as end-products; (iv) to determine if the products that are produced from biphenyl-grown cells are same as those produced by cells grown on other carbon sources; and (v) to isolate and characterize the first enzyme of the (chloro)biphenyl degradation pathway, and if possible to determine the effect of temperature on the products of transformation of 2,4'-diCB by this enzyme.

Chapter 2

Isolation, Characterization, and Identification of Biphenyl-Utilizing Psychrotolerant strain IA3-A that Cometabolize Chlorinated Biphenyls as *Hydrogenophaga* species

2.1 Introduction

Aerobic degradation of monoCBs and diCBs, and several triCBs by mesophilic microorganisms has been widely reported (Kim and Picardal, 2001; Bedard and Haberl, 1990; Tucker *et al.*, 1975), but relatively few studies have described aerobic degradation of biphenyl or PCBs by psychrotolerant (also known as psychrotrophic) or psychrophilic microorganisms. The earliest study reported that several psychrotrophic bacterial strains failed to mineralize 2-monoCB (Whyte *et al.*, 1995), but information on the extent of removal of the compound and identity of the metabolic products was not provided. Since then, only two studies reported the isolation of cold-adapted PCB-degrading bacteria (Master and Mohn, 1998; Mohn *et al.*, 1997). Though, Williams and May (1997) demonstrated that aerobic degradation of PCBs occurred at low temperature in sediments contaminated with PCBs, but there was no indication of the identity of the microorganisms involved. Therefore, much is not known about PCB-degrading psychrotolerant microorganisms. In contrast, several studies have described cold-adapted microorganisms that are capable of degrading other common environmental pollutants. Microbial degradation of phenol (Kotturi *et al.*, 1991), chlorophenol (Jarvinen *et al.*, 1994) and tetrachlorophenol (Mannisto *et al.*, 2001), C_5 - C_{12} and C_6 - C_{20} *n*-alkanes (Bej *et al.*, 2000; Whyte *et al.*, 1997), naphthalene (Whyte *et al.*, 1997; Whyte *et al.*, 1995), and toluene (Whyte *et al.*, 1997) has been demonstrated at low temperature.

There are few reports of biodegradation of aromatic compounds by bacterial species in the genus *Hydrogenophaga* (Kampfer *et al.*, 2005; Blumel *et al.*, 1998; Feigel and Knackmuss, 1993). A previous study isolated and identified a *H. pseudoflava* strain as a member of a PCB-degrading microbial community, but the bacterium was not characterized with respect to its ability to degrade PCBs (Joshi and Walia, 1995). Though, psychrotolerant strains of members of the genus *Hydrogenophaga* have been characterized (Brummer *et al.*, 2003; Rutter and Nedwell, 1994), none of these strains were shown to degrade PCBs or any aromatic compounds. In this regard, detailed investigation of aromatic compound degrading psychrotolerant strain belonging in the genus *Hydrogenophaga* is necessary.

This chapter describes a cold-adapted biphenyl-utilizing strain that was isolated from a long-term PCB-contaminated soil. Preliminary investigations in the present study indicate that biphenyl-grown cells of the cold-adapted strain degraded 2,3- or 2,4'-diCB, and few other aromatic compounds. However, 2,3- or 2,4'-diCB were not used as source of carbon and energy. Physiological and biochemical characterization of the bacterium was done. These, combined with the profile of cellular fatty acids were used to identify the strain to the genus level.

2.2 Materials and Methods

2.2.1 ***Source, enrichment, and isolation of biphenyl-utilizing bacteria.*** Five grams of soil samples that were collected from different locations of a PCB-contaminated site and an uncontaminated site, at the Pinetree Radar site north-west of the town of Stephenville (48.32°N, 58.33°W) in western Newfoundland, were transferred into 50 mL of minimal salt medium (MSM) containing biphenyl (0.5 %, w/v) placed in a 250 mL flasks. The flasks were incubated at 30 °C on a shaker at 200 rpm for 7 days. Then, a loop-full of the cultures were streaked on MSM solidified with 1.5 % (w/v) Bacto-agar (Difco, Detroit, MI, USA) (minimal salt agar, MSA) and biphenyl was supplied in the vapor form by crystals that were placed in the lid of the inverted plates. The cultures were incubated at 30 °C until visible colonies were observed. Morphologically different colonies that formed on the medium were selected and sub-cultured on MSA plates supplied with biphenyl crystals as above, followed by incubation at 30 °C. The purity of the cultures was confirmed by gram-staining after several single colony transfers on trypticase soy agar (TSA) (Difco, Detroit, MI, USA).

2.2.2 ***Growth medium.*** MSM was composed of the following (g L^{-1}): $(NH_4)_2SO_4$ (0.5), $MgSO_4.7H_2O$ (0.1), $Ca(NO_3)_2$ (0.075), plus vitamins (1 mL) and trace elements solution (10 mL) per litre of 40 mM phosphate buffer (KH_2PO_4 and Na_2HPO_4, pH 7.3) (Adriaens *et al.*, 1989). The vitamin solution contained 0.04 (mg L^{-1}) each of biotin, folic acid, riboflavin, nicotinic acid, pantothenic acid, and vitamin B12. The trace elements solution was made up of (g L^{-1}) $CaSO_4$ (0.2), $FeSO_4.7H_2O$ (0.2), $MnSO_4.H_2O$ (0.02), $NaMo.2H_2O$ (0.01), $CuSO_4$ (0.02), $CoSO_4.7H_2O$ (0.01), H_3BO_3 (0.005) and 200 µL of concentrated H_2SO_4 to prevent

precipitation of basic salts (McCullar *et al.*, 1994). The vitamins solution was filter sterilized prior to use. All other solutions and media were sterilized by autoclaving at 15 psi, 121 °C for 30 min.

Where applicable, cells were grown on biphenyl in order to induce biphenyl-degrading enzymes. Cells that were grown on TSA (24 h) were washed in sterile saline (0.85 %, w/v) or 0.05 M sodium phosphate buffer (pH 7.5), and the cells were resuspended in the same buffer. The cell suspension was inoculated into MSM (final volume, 500 mL) containing biphenyl (0.5 %, w/v) placed in a 1-L flask. The culture was incubated at 30 °C on a shaker at 200 rpm. Growth was monitored turbidometrically at 600 nm using a Shimadzu UV-260 spectrophotometer (Shimadzu Co. Kyoto, Japan). Cells were harvested during the exponential phase of growth by centrifugation (15,000 *g*, 4 °C, 15 min) using a Sorvall RC 5C Plus centrifuge (DuPont, Wilmington, USA), after removal of biphenyl crystals with sterile glass-wool. Cell pellets were washed twice in sterile saline (0.85 %, w/v) or 0.05 M sodium phosphate buffer (pH 7.5), and cells were resuspended in the same buffer.

2.2.3 ***Physiological characterization of biphenyl-utilizing bacteria.*** Morphologically different isolates were characterized microscopically and physiologically following standard protocols (Bergey, 1994). Cold-adaptation of the bacteria was confirmed by inoculating the isolates on TSA followed by incubation at 5 °or 7 °C. The most stable cold-adapted isolate, as determined by the ability to grow on biphenyl-supplemented MSA after a prolonged incubation (72 h) on TSA, was chosen for further investigation.

Tryptic soy agar-grown cells of the cold-adapted isolate were inoculated into 30 mL of tryptic soy broth (TSB) (Difco, Detroit, MI, USA) placed in a 125-mL flasks and the cultures were incubated at 4 °, 10 °, 15 °, 20 °, 25 °, 30 °, 35 °, 40 °, and 45 °C. Growth was determined turbidometrically at 600 nm with a Shimadzu UV-260 spectrophotometer. Alternatively, growth was determined by estimation of protein concentration after incubating cells in TSB or MSM containing biphenyl. Biphenyl-grown cells (30.7 - 47.9 mg protein L^{-1}) were inoculated into 15 mL of TSB or MSM placed in 50 mL flasks. Biphenyl that was already dissolved in acetone was added into the MSM to a final concentration of 0.1 % (w/v). The TSB and biphenyl-containing cultures were incubated in triplicates at 5 °, 10 °, 20 °, 30 °, and 35 °C on a shaker at 200 rpm for 72 – 336 h. Thereafter, biphenyl crystals were removed from biphenyl-containing cultures and cells were harvested as outlined in section 2.2.2. Protein concentration was determined by the method of Bradford (1976) with bovine serum albumin (BSA) as a standard.

Preliminary identification of the cold-adapted strain was carried out using the API 20E kits (bioMerieux Inc., St. Laurent, QE, Canada) by following the manufacturer's instruction. The bacterium, grown on tryptic soy broth agar (TSBA), was further identified by analysis of the profile of cellular fatty acids using the MIDI/Hewlett-Packard Microbial Identification System (Analytical Services, Williston, VT, USA). The identity of the bacterium was confirmed with reference to the Bergey's Manual of Determinative Bacteriology (Bergey, 1994). The final physiological tests that were carried out included denitrification of nitrate; growth on (50 or 100 mg L^{-1} of MSM) D-xylose, lactose, mannitol, formate, L-histidine, sucrose, maltose, D-fructose, *p*-hydroxybenzoate, pyruvate, succinate, acetate, or citrate; decarboxylation of lysine, arginine, or ornithine; oxidation-fermentation of maltose,

mannitol, and glucose; and hydrolysis of gelatine, and DNA (Kampfer *et al.*, 2005; Willems *et al.*, 1989).

2.2.4 ***Growth and oxygen uptake.*** Biphenyl-grown cells were suspended at an optical density ($OD_{600\ nm}$) of 0.2 in 100 mL of MSM placed in 250-mL flasks. Concentrated stock solution (2 μL) of 2,3- or 2,4-diCB (ULTRA Scientific North Kingstown, RI, USA) that was dissolved in acetone was added into the cultures to a final concentration of 250 μM. Controls were similar to the experimental treatments except that the cultures contained live cells and 2 μL of acetone only or heat-killed cells and 250 μM of each substrate. The cultures were incubated at 30 °C on a shaker at 200 rpm for 14 days. Growth was monitored by measurement of $OD_{600\ nm}$ and by estimation of colony forming units (CFU) on TSA.

The ability of the isolate to grow on various aromatic compounds was determined by transferring biphenyl-grown cells into 250-mL flasks containing 100 mL of MSM to give an initial $OD_{600\ nm}$ of 0.2. Each of the test aromatic substrates was added into experimental cultures to a final concentration of 5 mM. Positive-control treatments contained 5 mM of test substrates and 5 mM succinate in 100 mL of MSM. The cultures were incubated at 30 °C at 200 rpm on a shaker for 10 days. Growth was determined by estimation of protein concentration using the method of Bradford (1976) with BSA as a standard.

Rate of oxidation of various aromatic compounds was measured using a Clark-type oxygen electrode (Yellow Springs Instrument, Co., Yellow Springs, Ohio) connected to a 2 ml volume reaction chamber held at 30 °C. Biphenyl-grown cells were suspended in 0.05 M sodium phosphate buffer (pH 7.5) to an $OD_{600\ nm}$ of 2.0 as previously described. Then, 1.8

mL of 0.05 M sodium phosphate buffer (pH 7.5) was placed in the reaction chamber followed by the addition of 5 or 10 μL solution of each substrate dissolved in the same buffer for readily soluble substrates or a solution of the substrate dissolved in acetone for insoluble substrates. Final concentration of individual substrate in the reaction mixtures was 0.5 or 5 mM respectively. Reactions were initiated by transferring 0.2 mL of the cell suspension into the reaction chamber to achieve an initial $OD_{600\ nm}$ of 1.0. Oxygen uptake rates were corrected for endogenous respiration in the absence of substrate or in the presence of acetone only, a substrate that was not metabolized.

2.2.5 ***Analytical methods.*** Protein concentration was determined by the method of Bradford (1976) after releasing protein from cells by alkaline hydrolysis. Analysis of fatty acids in cell membrane was determined using high resolution gas chromatography and mass spectrometry (Analytical Services, Inc., VT, USA). The fatty acids profile of the unknown isolate was compared to the fatty acid profiles of known isolates in a database, which included numerous species of *Neisseria*, *Acidovorax*, *Pseudomonas*, *Arcobacter*, *Campylobacter*, *Wollinella*, and *Hydrogenophaga*.

Formation of metabolic intermediates from 2,3- or 2,4'-diCB was confirmed by preparative TLC. Fifty milliliter of culture supernatant was extracted twice with equal volume of hexane. Thereafter, the aqueous phase was acidified to pH 2.0 – 3.0 with 1 M HCl and extracted twice with two equal volume of ethyl acetate. The hexane and ethyl-acetate phases were dried over anhydrous sodium sulfate and evaporated separately using a rotovapor set at 70 ° or 78 °C, respectively. The residues from each of the extracts were

dissolved in 1 mL of hexane or ethylacetate. The solution was spotted on a F_{254} silica gel plate using a microsyringe. The plates were developed in a dioxane-benzene-acetic acid (90 : 20 : 4) solvent system (Furukawa *et al.*, 1978). The plates were viewed under UV and photographed using a ChemiImager (Alpha Innotech Co., San Leandro, CA, USA). The spots were compared with those of authentic standards of 2-, 4-CBA (ULTRA Scientific North Kingstown, RI, USA) or 2,3-diCBA (CBA: chlorobenzoic acid) (Crescent Chemical Co., New York, USA), which are the expected metabolic end-products of 2,4′- or 2,3-diCB, respectively. Then, the spots were scrapped off the plate and extracted separately into ethyl-acetate. The ethyl-acetate was evaporated to dryness as outlined above and the residue was dissolved in methanol. The methanol solution was then analyzed by UV spectrometry. The spectra were compared with the available authentic standards.

2.3 Results

2.3.1 ***Biphenyl-utilizing bacterial isolates.*** Incubation of soil samples in MSM containing biphenyl at 30 °C for 7 days and subsequent subculturing on MSA with biphenyl, and TSA yielded seven morphologically distinct bacterial colonies that were capable of using biphenyl as a sole source of carbon and energy. Six of the isolates designated as A1A, A1B, A1C, A1C3, A1F, and IA3-A were isolated from soils collected from a PCB-contaminated site. The only isolate from a non-contaminated site was designated as CON1. All the isolates formed distinct colonies within 72 - 120 h on MSA with biphenyl at 30 °C and they exhibited distinct physiological characteristics (Table 2.1). However, only strain A1C and IA3-A grew on TSA that was incubated at 5 ° or 7 °C. Pure cultures were obtained after several transfers on TSA and this was confirmed by microscopic examination. Repeated attempts to maintain strain A1C on solid or liquid media with biphenyl after subculturing on TSA were unsuccessful, but the strain could be maintained occasionally on solid or liquid media with biphenyl when it is subcultured from TSA plate that is supplied with biphenyl crystals. Strain IA3-A could be maintained continuously on TSA without losing the ability to grow on biphenyl. Therefore, strain IA3-A was chosen for further investigations.

Microscopic examinations of strain IA3-A showed that cells occurred singly or in pairs. It formed circular, smooth, opaque, convex, and pale-yellow colonies with entire margin when cultured on nutrient agar. In addition to its previously described physiological characteristics (Table 2.1), strain IA3-A denitrified nitrate anaerobically to nitrogen which was confirmed by formation of gas bubbles in inverted durham tubes in the test medium; it hydrolyzed gelatine but failed to hydrolyze DNA; it utilized sucrose, mannitol, D-xylose, pyruvate, succinate, acetate, and citrate, but it did not utilize lactose, D-fructose, maltose, *p*-

hydroxybenzoate, or formate. It did not produce acid from oxidative-fermentative medium containing glucose, mannitol, or maltose. It was negative for lysine, arginine, and ornithine decarboxylase. It grew on TSB at temperatures between 4 ° to 40 °C, and growth was not observed at 45 °C. Its optimum temperature on TSB was 30 °C (Lambo and Patel, 2006b).

The protein concentrations of cells of strains IA3-A that were cultured in TSB or in the presence of 0.1 % (w/v) biphenyl at 5 °C increased by 3.4 ± 0.7 and 0.1 ± 0.3 mg L^{-1} after 336 h, respectively. Although, growth was observed at all the temperatures tested, protein concentrations were significantly higher in cells subcultured in TSB than those subcultured in biphenyl-supplemented liquid medium (Fig. 2.1). The effect of low temperature on growth was more severe for cells in the presence of biphenyl than in TSB. For both carbon sources, incubation at 30 °C resulted in the highest mean amount of protein whereas the lowest amount was at 5 °C. There was high variability in protein concentrations in cells cultured on TSB when compared to those cultured on biphenyl. However, in a separate study maximum optical density readings was observed in cells cultured on TSB at 30 °C when compared to those cultured on TSB at other temperatures between 5 - 45 °C.

The profiles of fatty acids in strain IA3-A showed that it contained fatty acids with 10 to 18 carbon atoms (Table 2.2.). The predominant fatty acids include *cis*-7-hexadecenoic acid and/or 2-hydroxy-iso-pentadecanoic acid (47.75 %), hexadecanoic acid (27.4 %), and *cis*-7-octadecenoic acid (11.78 %). Results of cellular fatty acids analysis suggests that it was most closely related to *H. taeniospiralis* with a similarity index (SI) of 0.669. The similarity index was lower when the fatty acids profile of strain IA3-A was compared to those of *Acidovorax konjaci*, *Hydrogenophaga pseudoflava*, *Acidovorax avenae*, and some other species in the database (Table 2.3).

A comparison of the substrate utilization and biochemical profiles of strain IA3-A to species in the genus *Hydrogenophaga* showed that it is more similar to *H. taeniospiralis* than other species in this genus (Table 2.4). But unlike previously described strain of *H. taeniospiralis* (Willems *et al.*, 1989), strain IA3-A contained 3-hydroxydecanoic acid; it utilized sucrose; it exhibited a weak catalase reaction; and it did not hydrolyze urea. Attempts to identify strain IA3-A using the API 20E kit did not give a conclusive result and only four of the tests (VOGES-PROSKAUER, gelatinase, production of acid from glucose, reduction of NO_3 and production of N_2) were positive after 48 h. The bacterial strain was designated as *Hydrogenophaga* sp. IA3-A.

2.3.2 ***Cometabolism of 2,3- and 2,4'-diCB.*** An increase in the turbidity of cultures incubated with 2,3- or 2,4'-diCB indirectly suggests that cells of strain IA3-A grew on both substrates (Fig. 2.2). However, estimation of the CFU in the cultures indicates that growth did not occur and that most of the cells were not viable (Fig. 2.3). Concentration-dependent decrease of CFU in the cultures showed that the substrates or the metabolic intermediates produced from the substrates were likely toxic to the cells. The effect of 2,3-diCB on cell viability was more pronounced at all the concentrations tested when compared to the effect of 2,4'-diCB on cell viability. The lack of growth on 2,3- or 2,4'-diCB indicates that cells of *Hydrogenophaga* sp. IA3-A only cometabolized the compounds. The optimal temperature, pH, and substrate concentration for oxidation of 2,4'-diCB by cells of strain IA3-A in 0.05 M sodium phosphate buffer was 30 °C, pH 7.5, and 0.25 mM 2,4'-diCB, respectively (Fig. 2.4).

2.3.3 ***Formation of metabolic intermediates.*** *Hydrogenophaga* sp. IA3-A transformed each of the substrates into metabolic intermediates that could be detected by TLC. Several intermediates were detected in the extracts of cultures incubated with 2,3-diCB (Fig. 2.5). One of the intermediates was present at low intensity and it had a Rf value (0.8 ± 0.04) and UV spectra that were identical to those of authentic standard of 2,3-CBA (Fig. 2.6 and Table 2.5). Several intermediates were also detected by TLC in the extracts of the cultures incubated with 2,4'-diCB (Fig. 2.5 and Table 2.6). The extracts of these cultures also contained metabolic products that were not resolved on the TLC plate. Two unresolved metabolic products migrated on the plate at similar rates as authentic standards of 2- and 4-CBA, but the UV spectra of the products were not identical to those of the standards.

2.3.4 ***Oxidation uptake with various aromatic compounds.*** Cells of strain IA3-A grown on biphenyl were able to utilize various aromatic compounds at 30 ^{o}C (Table 2.7). The most abundant growth occurred on protocatechuate and phloroglucinol, whereas a few of the substrates that were tested did not support the growth of the strain. .

Many of the aromatic compounds that were tested were also oxidized at different rates (Table 2.8). The highest rate of oxygen uptake (223 ± 7 nmol min^{-1} mL^{-1} mg $protein^{-1}$) was recorded in the presence of catechol, whereas the chlorine and methyl substituted analogues of the compound stimulated relatively lower uptake rates. The rate of oxidation of *p*-hydroxybenzoate was much lower, and oxygen uptake rate was not different from the controls in the presence of resorcinol, phloroglucinol, 4-chloro-*m*-cresol, and toluene.

Benzene caused a moderate uptake of oxygen, whereas 2,4'- diCB which was present at a much lower concentration was oxidized at a relatively high rate.

Table 2.1. Some morphological and physiological characteristics of biphenyl-utilizing bacteria isolated from PCB-contaminated and non-contaminated soils.

	Bacterial isolates						
Characteristics	A 1A	A1B	A1C	A1C3	A1F	CON1	IA3-A
Growth at 5 °C[a]	-	-	+	-	-	-	+
Gram-stain	-	-	-	-	-	-	-
Shape	rods	rods	rods	rods	rods	rods	rods
Motility	+	+	+	+	+	+	+
Endospores[b]	+	+	-	-	+	+	+
Catalase	+	+	+	+	+	+	+[c]
Oxidase	-	-	+	-	+	-	+
Reduction of NO_3^-	-	+	+	-	-	-	+
Production of N_2[d]	-	-	+	-	-	-	+
Utilize citrate	-	-	-	-	-	-	-
Hydrolysis of lipid	-	-	-	-	-	-	-
Hydrolysis of casein	-	-	-	-	-	-	-
Hydrolysis of starch	-	+	-	+	+	+	-
Production of indole	-	+	-	-	-	-	-
Production of H_2S	-	+	-	-	-	-	-
Acid / gas from							
Glucose	+/-	+/+	+/-	+/-	+/-	+/-	+/-[e]
Lactose	-/+	-/+	-/-	-/+	-/+	+/+	+/-
Maltose	+/+	+/+	-/+	+/+	+/+	+/+	-/-
Sucrose	+/+	+/-	+/+	+/-	+/+	+/+	+/-

a, incubation on TSA for 120 h (similar results were obtained at 7 or 10 °C); *b*, observations were recorded after 24 or 48 h; *c*, weak positive (similar results after several trials); *d*, formation of gas bubbles in inverted durham tubes placed in test medium; *e*, positive reaction for acid production from glucose was observed after 3 weeks of incubation.

Table 2.2. Profile and percent composition of fatty acids in the cell membrane of *Hydrogenophaga* sp. IA3-A

Fatty acid[a]	Percent composition	ECL[b]	ECL deviates[c]
10 : 0 3OH	2.03	11.425	0.003
12 : 0	3.07	12.000	0.000
14 : 0	1.86	14.000	0.000
15 : 1 w6c	0.67	14.859	0.003
15 : 0	3.43	15.000	0.000
16 : 1 w7c / 15 : 0 iso 2OH	47.75	15.822	0.000
16 : 0	27.40	16.000	0.000
17 : 0 CYCLO	0.80	16.889	0.001
17 : 0	1.22	16.999	-0.001
18 : 1 w7c	11.78	17.823	0.000

[a]Fatty acids were identified by high resolution mass spectrometry and by comparison of the unknown to standards in the database; 10 : 0 3OH, 3-hydroxydecanoic acid; 12 : 0, dodecanoic acid; 14 : 0, tetradecanoic acid; 15 : 1 w6c, *cis*-6-pentadecanoic acid; 15 : 0, pentadecanoic acid; 16 : 1 w7c, *cis*-7-hexadecanoic acid; 15 : 0 iso 2OH, 2-hydroxy-iso-pentadecanoic acid; 16 : 0, hexadecanoic acid; 17 : 0 CYCLO, cycloheptadecanoic acid; 17 : 0, heptadecanoic acid; 18 : 1 w7c, *cis*-7-octadecenoic acid.

[b]ECL, equivalent chain lengths

[c]ECL deviates, indicates the difference between the elution time of fatty acids in unknown strain when compared to the elution time of known standards in the database.

Fatty acids were identified by high resolution mass spectrometry and by comparison of the unknown to standards in the database.

Table 2.3. Similarity of the cellular fatty acids in the cell membrane of *Hydrogenophaga* strain IA3-A to the fatty acids of some bacterial isolates in the MIDI/Hewlett Packard Microbial Identification System database

Bacterial strain	Similarity index (SI)[a]	Distance
H. taeniospiralis	0.669	3.187
H. pseudoflava	0.451	ND
Acidovorax konjaci	0.507	ND
Acidovorax avenae	0.399	ND
A. avenae subspecies citrulli	0.399	ND
A. avenae subspecies *cattleyae*	0.302	ND
Acidovorax facilis	0.396	ND
Arcobacter cryaerophilus	0.203	6.187
Neisseria sicca	0.115	64.225

ND, not determined

[a]SI, The Similarity index is a numerical value which expresses how closely the fatty acid composition of an unknown compares with the mean fatty acid composition of the strains used to create the entry listed as its match. An SI of 0.500 or higher with a separation of 0.100 between the first and second choice is considered a good library comparison. If the SI is between 0.300 and 0.500 with a separation of 0.100 between the first and second choice, this may be a good match, but atypical strain. A SI lower than 0.300 indicate that either the species is not represented in the database or the species is most likely the closest related species (Analytical Services Inc., 2004).

Table 2.4. Comparison of strain IA3-A to other species in the genus *Hydrogenophaga*

Characteristics	Strain IA3-A	1	2	3	4	**5**	6	7
Utilization of								
Sucrose	+	-	-	+	+	-	+	-
D-Fructose	-	-	-	+	+	-	+	-
Formate	-	-	-	-	-	-	+	-
Mannitol	+	-	-	+	+	+	-	+
Maltose	-	-	-	+	+	-	-	-
Lactose	-	nt	nt	-	+	-	-	nt
L-Histidine	-	+	-	+	+	-	-	-
D-xylose	+	-	-	-	+	+	-	-
p-Hydroxybenzoate	-	+	+	nt	+[a]	-	nt	+
Succinate/pyruvate	+/+	nt/+	nt/+	nt	+/+	+/+	nt	nt/-
Denitrification	+	-	-	-	+	+	-	-
Catalase	+[b]	+	+	nt	-	-	nt	nt
Urease	-	nt	nt	+	-	+[b]	nt	nt
DNAse	-	nt	nt	nt	-[c]	-	nt	nt
Reduction of nitrate	+	+	+	+	+	+	-	+
Growth on MacConkey agar	-	+[b]	+[b]	nt	nt	nt	nt	nt
Presence of 10 : 0 3OH	+	-	-	+	+	-	-	nt
Acid from O-F medium containing[d]								
Glucose	-	nt	nt	nt	-	-	+[a]	nt
Maltose	-	nt	nt	nt	-	-	nt	nt
Decarboxylation of A-L-O[e]	-	nt	nt	nt	-	-	nt	nt

Strains: 1, *H. defluvii*; 2, *H. atypical*; 3, *H. flava*; 4, *H. pseudoflava*; **5, *H. taeniospiralis***; 6, *H. palleroni*; 7, *H. intermedia.*

a, positive only for some strains; *b*, weak reaction or growth; *c*, absent in almost all strains; *d*, O-F indicates oxidative-fermentative; *e*, A-L-O indicates arginine, lysine, and ornithine; nt, not tested. Data for the other seven strains were obtained from Kampfer *et al.* (2005); Contzen *et al.* (2000); and Willems *et al.* (1989).

Table 2.5. Detection of intermediates in culture extracts of *Hydrogenophaga* sp. IA3-A incubated with 2,3-DiCB

	Rf value of intermediates	Rf value of authentic standards
Spot no.	Experimental	2,3-diCBA
1	0.06±0.002	0.75±0.04[a]
2	0.08±0.001	
3	0.22±0.001	
4	0.37±0.001	
5	0.69±0.001	
6	0.79±0.004	

TLC was performed using a dioxane-benzene-acetic acid (90 : 20 : 4) solvent system

N = 3 ± SD;

[a]N = 2 ± SD

ND, not determined

Table 2.6. Detection of intermediates in extracts of cultures of *Hydrogenophaga* sp. IA3-A incubated with 2,4'-DiCB

	Rf value of intermediates		Rf value of authentic standards		
Spot no.	Experimental	Control	2,4'-DiCB	2-CBA	4-CBA
1	0.06±<0.001	0.97±0.01	0.95±0.01	0.89±0.04	0.91±0.03
2	0.22±0.01				
3	0.36±0.01				
4	0.46±0.01				
5	0.58±0.06				
6	0.68±0.04				
7	0.72±0.02				
8	0.94±0.01				

TLC was performed using a dioxane-benzene- acetic acid (90 : 20 : 4) solvent system.

N = 3 ± SD

Table 2.7. Growth of *Hydrogenophaga* sp. IA3-A on selected aromatic compounds

	Biomass yield (mg protein mL^{-1})	
	Substrate	Substrate + Succinate
Catechol	1.91 ± 0.02	8.97 ± 4.54
4-Methylcatechol	0.14 ± 0.02	NT
Protocatechuic acid	4.48 ± 0.02	3.21 ± 0.66
Phloroglucinol	4.30 ± 1.61	6.23 ± 0.29
Resorcinol	0.53 ± 0.03	NT
4-Chlororesorcinol	0.29 ± 0.09	NT
Para-hydroxybenzoate	0	0.03 ± 0.14
Naphthalene	0	0.22 ± 0.1
2,4-Dichlorophenoxyacetate	0	3.12 ± 0.66

N = 3 ± SD

Reaction conditions: 5.0 mM of substrate in MSM or 5.0 mM of substrate plus 5.0 mM of succinate in MSM incubated at 30 °C for 10 days.

NT, not tested

Table 2.8. Specific rate of oxidation of different aromatic compounds by biphenyl-grown cells of *Hydrogenophaga* sp. IA3-A

Substrates[a]	Oxygen consumed [nmol of O_2 min^{-1} mL^{-1} mg $protein^{-1}$]
Catechol	229.88 ± 6.81
4-Chlorocatechol	36.86 ± 6.82
4-Methylcatechol	32.57 ± 2.19
Protocatechuic acid	7.60 ± 6.51
Phloroglucinol	0.0
Resorcinol	0.0
Para-hydroxybenzoate	9.07 ± 4.40
4-Chloro-*m*-cresol	0.0
Toluene	0.0
Benzene	22.07 ± 13.80
2,3-Dichlorobiphenyl	18.51 ± 16.48[b]
2,4'-Dichlorobiphenyl	35.34 ± 6.01[b]
Biphenyl	197.7 ± 28.6 (57.16 ± 8.66)[c]
Acetone	20.30 ± 2.42[d]

N = 4 ± SD

[a]Final concentration or amount of substrates: 2,3-dichlorobiphenyl, 0.5 mM; 2,4'-dichlorobiphenyl, 0.5 mM; toluene, 0.05 % (v/v); benzene, 0.05 % (v/v); all other substrates were present at a final concentration of 5.0 mM. Temperature of the reaction chamber was held at 30 °C.

[b]N = 3 ± SD

[c]Value in parentheses is rate of oxidation of 0.5 mM biphenyl

[d]Oxygen uptake value in the presence of (10 µL) acetone was similar to uptake value with cells only.

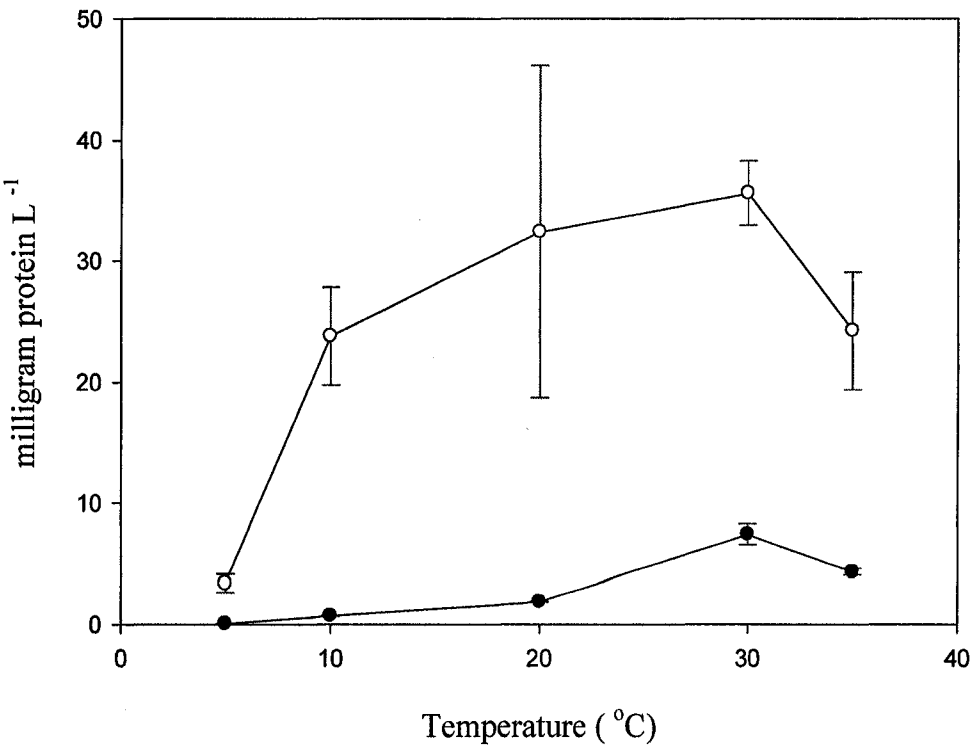


Fig. 2.1 Growth (mg protein L^{-1}) of cells of *Hydrogenophaga* strain IA3-A incubated in TSB (○) or MSM containing 0.1 % (w/v) biphenyl (●) at different temperatures. Data points are mean of three separate experiments. Error bars indicates standard deviation.

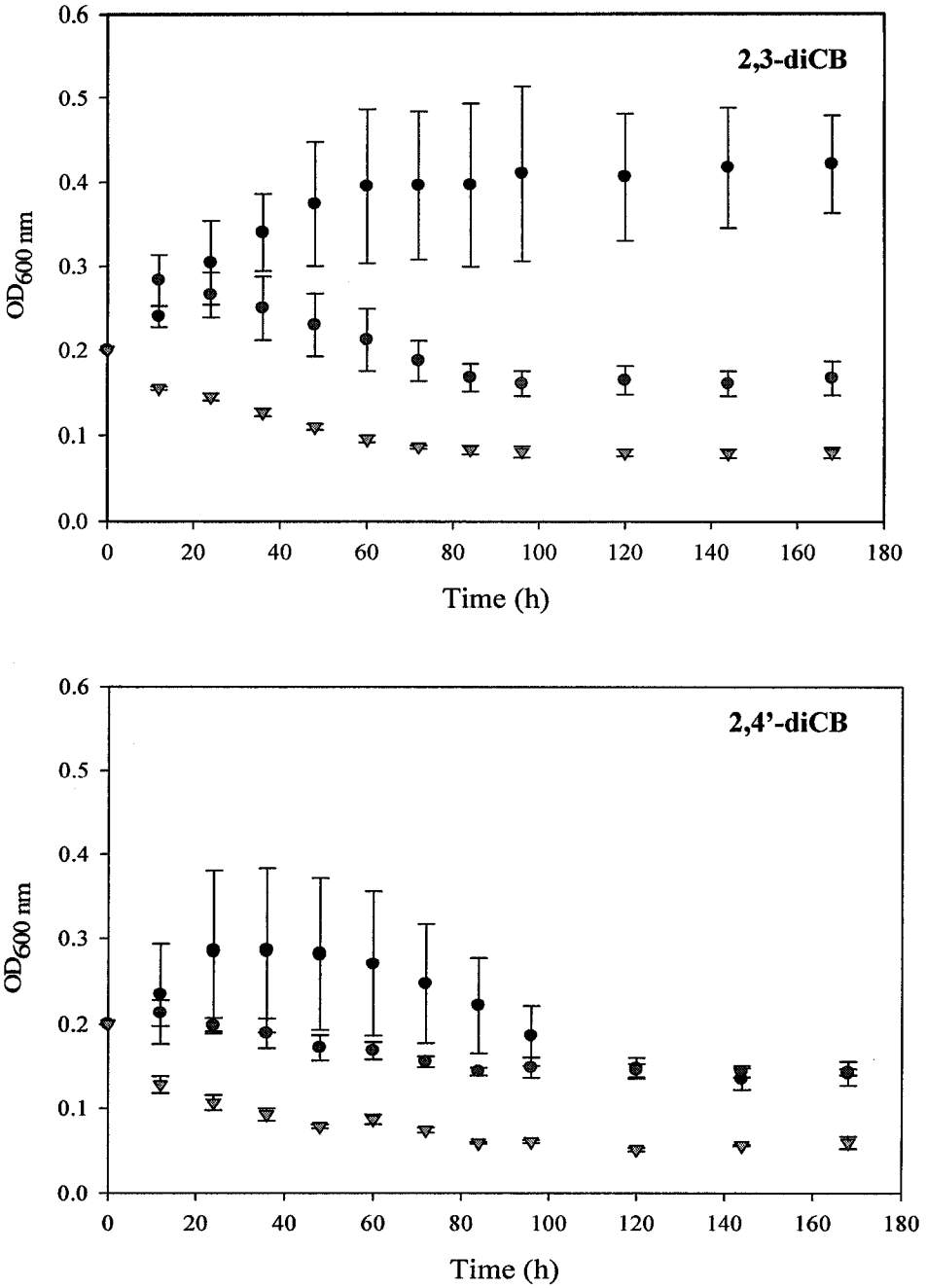


Fig. 2.2. Change in optical density of cultures containing 250 μM of substrate and live cells (●); cultures containing 250 μM of substrate and killed-cells (●); and cultures containing live cells only (▽). Data points are mean of three separate experiments. Error bars indicates standard deviation.

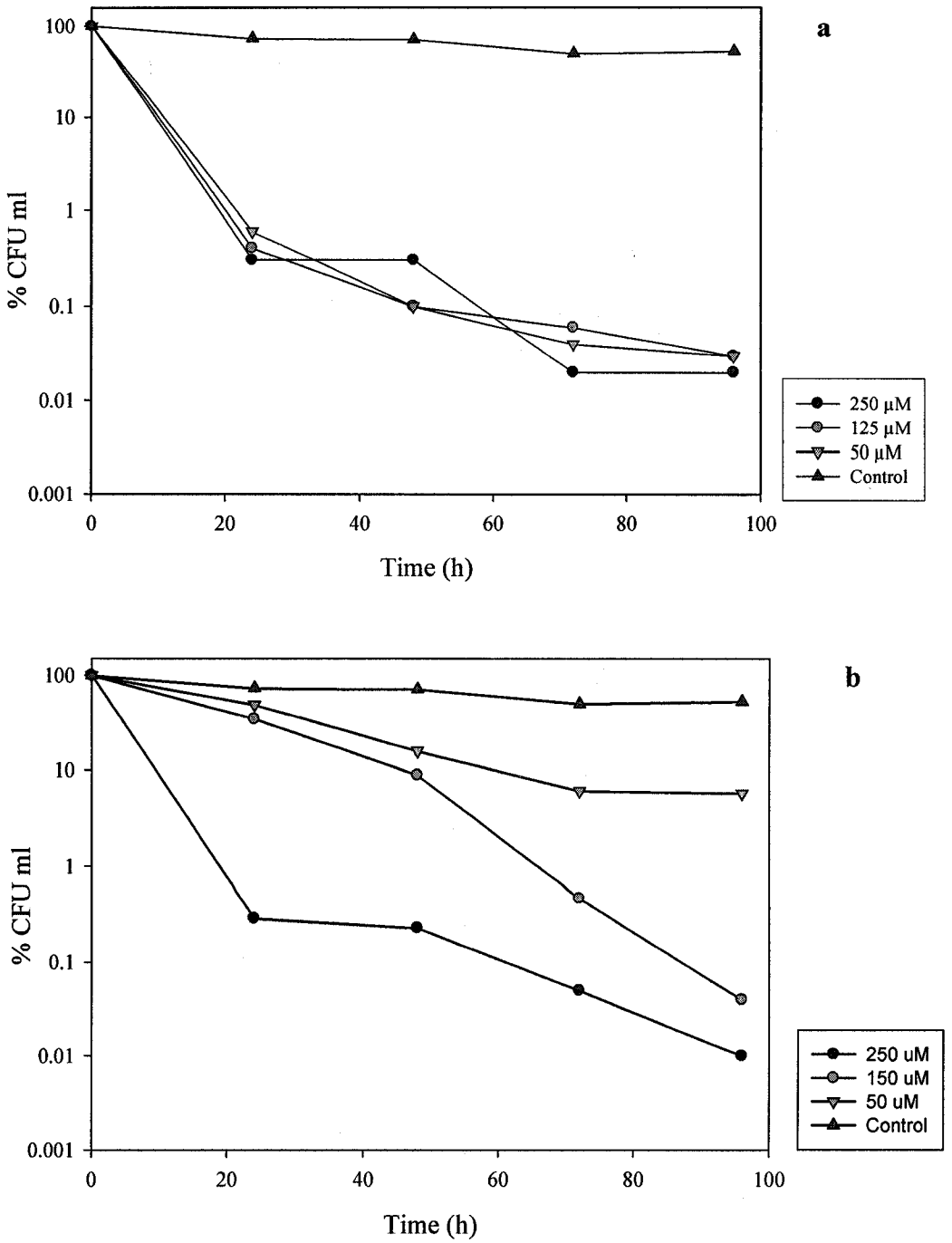


Fig. 2.3. Change in numbers of viable cells in cultures incubated with different concentrations of 2,3'-DiCB (**a**) or 2,4'-DiCB (**b**). Control cultures contained MSM and live cells only. Data points are mean of three separate experiments.

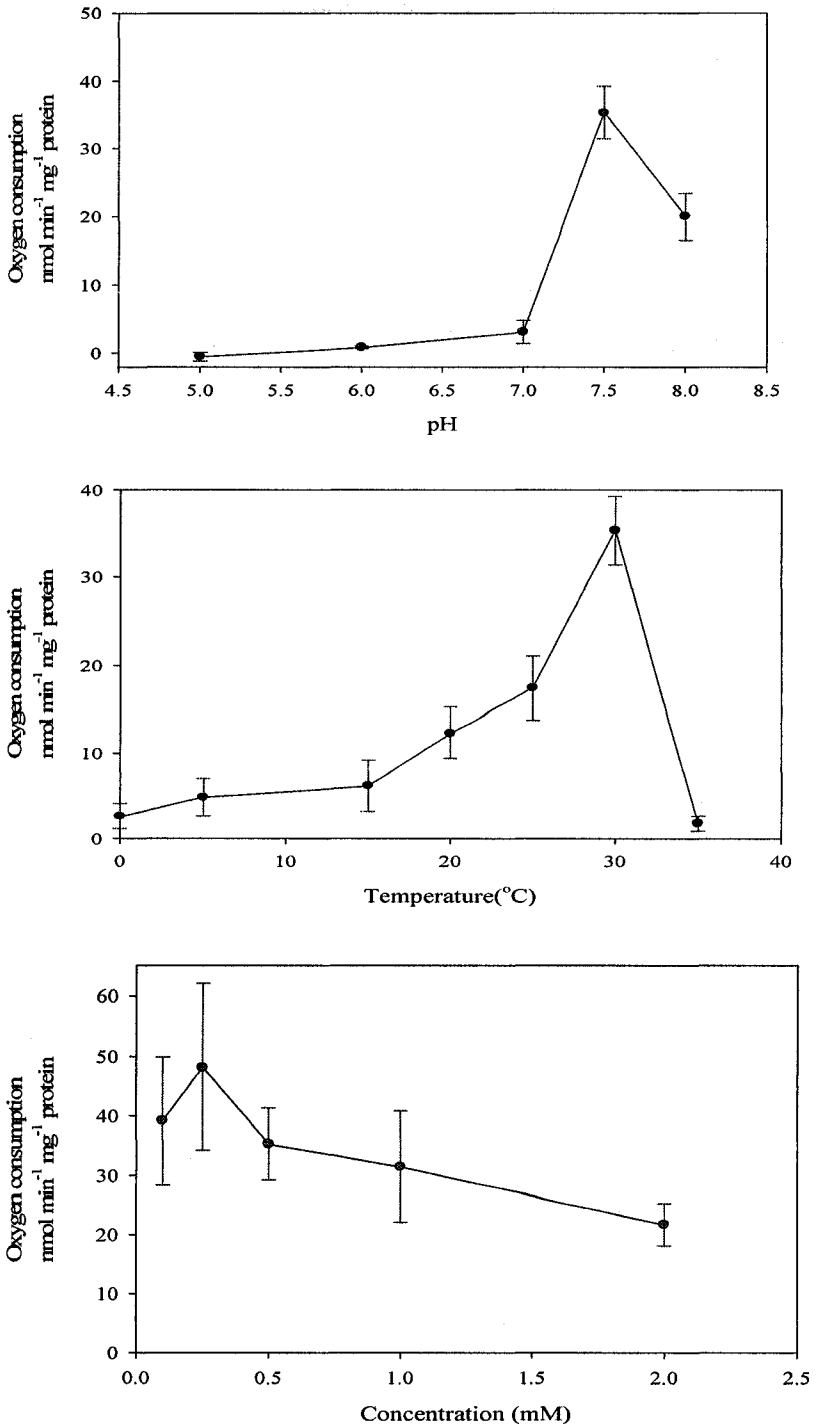


Fig. 2.4. Effect of pH, temperature, and substrate concentration on the oxidation of 2,4'-diCB by *Hydrogenophaga* sp. IA3-A. Data points are mean of three determinations, and error bars indicates standard deviation.

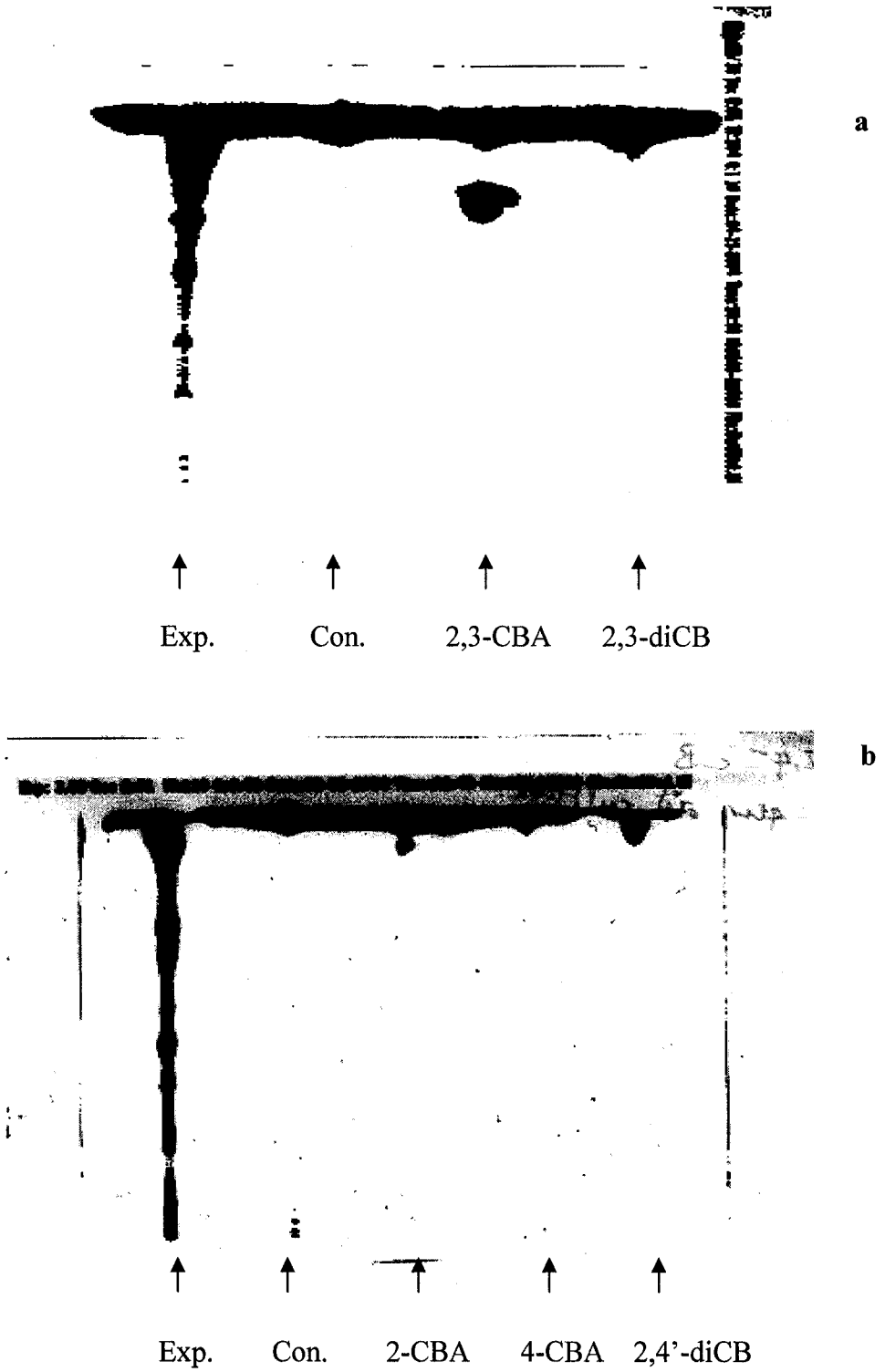


Fig. 2.5. Formation of metabolic products from 2,3- or 2,4'-diCB by *Hydrogenophaga* sp. IA3-A incubated at 30 °C for 14 days. **a**, TLC plate spotted with extracts of 2,3-diCB-containing cultures, standards of 2,3-diCBA and 2,3-diCB; **b**, TLC plate spotted with extracts of 2,4'-diCB-containing cultures, standards of 2-, 4-CBA, and 2,4'-diCB; Exp., live cells plus 2,3- or 2,4'-diCB; Con., killed cells plus 2,3- or 2,4'-diCB.

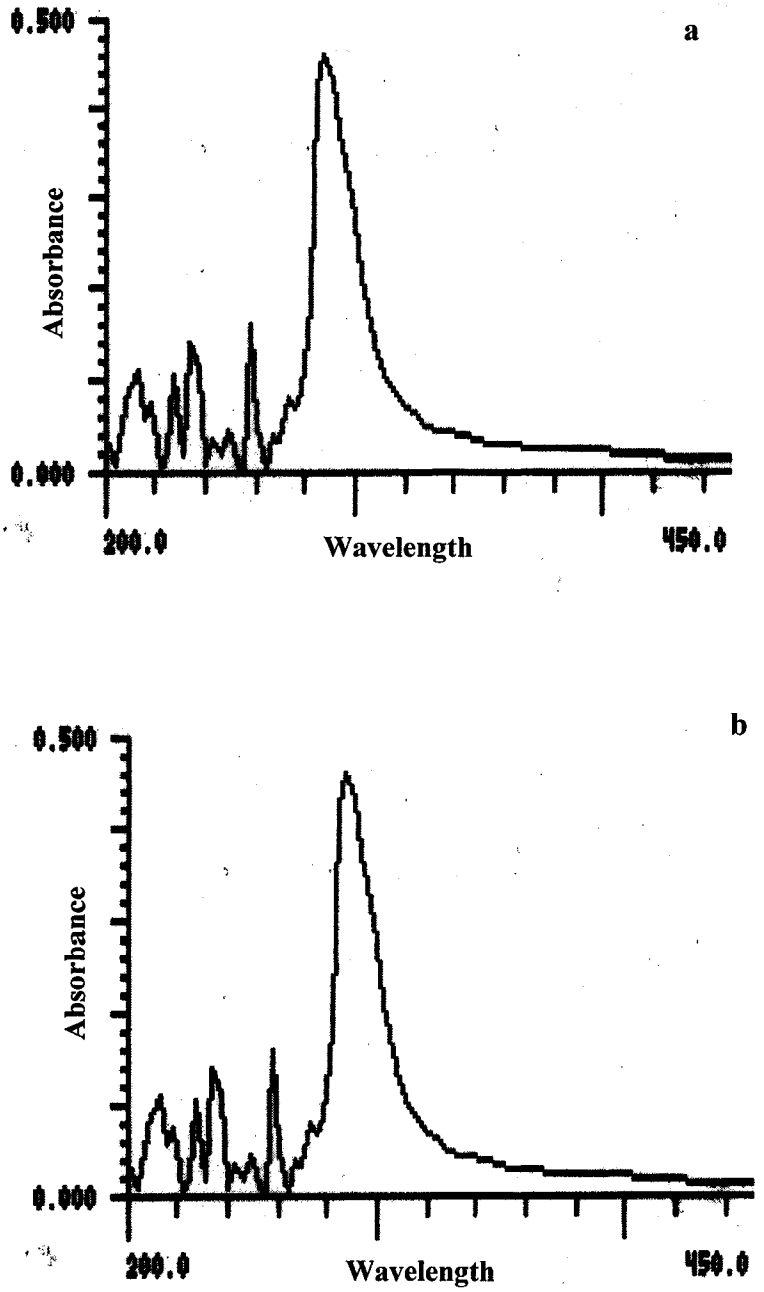


Fig. 2.6. UV-visible spectra of authentic standard of 2,3-diCBA (**a**) and a metabolic product that was extracted from spot no. 6 (**b**) (see Table 2.5) of the TLC plate spotted with extracts of cultures of strain IA3-A incubated with 2,3-diCB.

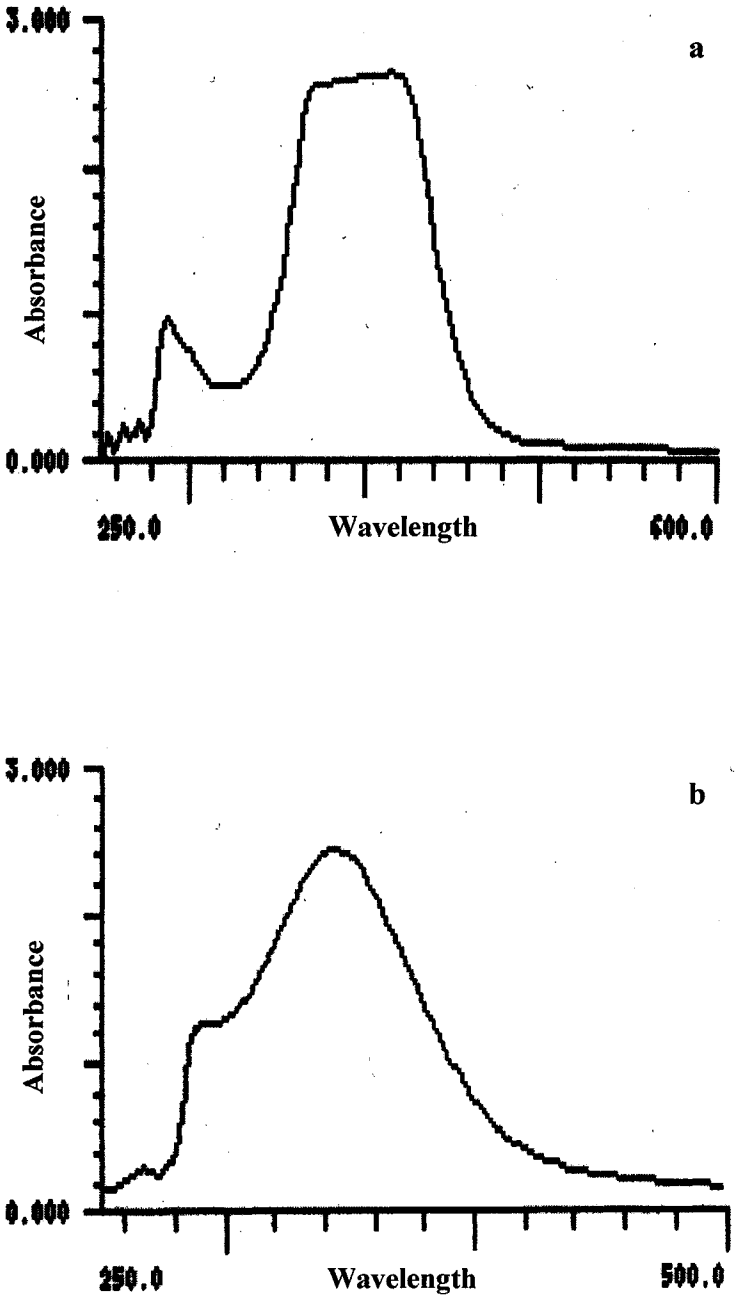


Fig. 2.7. UV-visible spectra of supernatant of cultures of *Hydrogenophaga* sp. IA3-A incubated with 2,4'-diCB at 30 °C for 14 days. **a**, spectra of supernatant at neutral pH; **b**, spectra of supernatant at acidic pH.

2.4 Discussion

The isolation of several biphenyl-utilizing bacterial strains from PCB-contaminated soils suggested that the exposure of bacteria in the soils to PCBs likely resulted in the selection of biphenyl-degrading microorganisms. Higher rate of mineralization of biphenyl has been observed for PCB-contaminated soil than uncontaminated soils, suggesting for *in situ* selection for biphenyl mineralizing organisms (Mohn *et al.*, 1997). The isolation of biphenyl-utilizing strain CON1 from uncontaminated soils in the present study also support the conclusion from previous studies that biphenyl-degrading bacteria are widespread and that enrichment of biphenyl-degrading bacterial strains from soils does not require exposure to PCBs (Mohn *et al.*, 1997; Chung *et al.*, 1994).

The inability of biphenyl-utilizing strains A1A, A1B, A1C3, A1F, and CON1 to grow on TSA at low temperatures suggest that these strains were mesophilic because they grew on TSA or biphenyl at 30 °C. This is in agreement with the knowledge that PCB-contaminated soils from temperate regions had fewer culturable heterotrophs which grew at 7 °C than at 30 °C (Mohn *et al.*, 1997). Several attempts to maintain TSA-grown cells of cold-adapted strain A1C on biphenyl-supplemented medium were not successful, but the strain grew occasionally on biphenyl-supplemented medium when it is cultured previously on TSA plates containing biphenyl crystals. In addition, strain A1C degraded 2,4'-diCB and formed metabolic products at 30 °C but it failed to degrade the same compound at 10 °C. These data suggest that an unstable genetic element might be involved in the degradation of (chloro)biphenyl in strain A1C, and that the product(s) of this element were not active at low temperature. In contrast to strain A1C, strain IA3-A could be maintained continuously on TSA without losing the ability to grow on biphenyl. In agreement with the definition of

Morita (1975), strain IA3-A is psychrotrophic (also known as psychrotolerant) because it grew on TSA at low temperatures and it had an optimum growth temperature that was above 20 °C.

The profile of cellular fatty acids and biochemical and morphological characterization suggest that strain IA3-A belong to the genus *Hydrogenophaga.* As with the type strains of all *Hydrogenophaga* species (Kampfer *et al.*, 2005; Contzen *et al.*, 2000; Willems *et al.*, 1989), strain IA3-A contained pentadecanoic acid, hexadecanoic acid, *cis*-7-octadecenoic acid, cycloheptadecanoic acid, *cis*-7-hexadecanoic acid and/or 2-hydroxy-iso-pentadecanoic acid. It also contained tetradecanoic acid like most of the type strains of the genus *Hydrogenophaga.* In contrast to previously characterized strains of the genus *Hydrogenophaga*, strain IA3-A did not contain 3-hydroxyoctanoic acid. It is possible that exposure of the bacterium to PCBs or other pollutants in the soils resulted in this variation. Exposure of a bacterial strain to aromatic compounds caused the alteration of its membrane fatty acids (Tsitko *et al.*, 1999). Thus, the absence of 3-hydroxyoctanoic acid in the cell membrane of strain IA3-A is not surprising, especially with the knowledge that strain IA3-A is most closely related to *H. taeniospiralis* (Table 2.3), which is known to contain relatively low amount of 3-hydroxyoctanoic acid (Willem *et al.*, 1989; Kampfer *et al.*, 2005). Its ability to reduce nitrate and its inability to utilize formate indicates that strain IA3-A is physiologically similar to most of the type strains of the genus *Hydrogenophaga* (Kampfer *et al.*, 2005; Contzen *et al.*, 2000; Willems *et al.*, 1989).

The isolation of *Hydrogenophaga* strain IA3-A from temperate soils is not unexpected. Studies have reported the isolation or presence of psychrotolerant *Hydrogenophaga* species in samples from permanently or seasonally cold environments (Brummer *et al.*, 2003; Rutter

and Nedwell, 1994). Strain IA3-A grew better on TSB than on biphenyl. On each medium, the concentration of cellular protein was most abundant at 30 °C. Effect of low temperature on growth was more pronounced in the presence of biphenyl than in TSB. Several factors could be responsible for the decreased growth at low temperatures. These include reduced growth rate and limited substrate uptake (Nedwell and Rutter, 1994). The difference in protein concentration between the cells that were grown at low temperatures on TSB and biphenyl could be explained in part by the difference in the nature of the two media. Yumoto *et al.* (2004) noted that adaptation following a shift from higher to lower temperature suggest a transition in physiological state that require certain nutritional conditions (e.g. amino acids, vitamins). The authors observed that, after a shift from 11 ° to 5 °C, cells of facultative psychrophile *Cobetia marina* L-2 had a significantly higher growth rate when they were grown on complex medium than when they were grown on defined medium.

The increase in turbidity of the cultures of strain IA3-A incubated with 2,3- or 2,4'-diCB did not correspond to an increase in numbers of CFU. After 24 h, the numbers of CFU in cultures with 250 μM of each substrate was less than 1 % of the initial population (Fig. 2.3). Similar results were obtained for all the cultures that were incubated with lower concentrations of 2,3-diCB, but the effect of 2,4'-diCB on cell viability was dependent on the concentration of the substrate. These results could be explained in part by the potential toxicity of the substrates to cells. Some chlorinated biphenyls including 2,3'-diCB were shown to be toxic to bacterial cells (Chu *et al.*, 1997). It is also possible that the products of transformation of these substrates were toxic to cells. A previous study concluded that transformation products of 2,3'-diCB and some other PCBs were toxic to cells (Camara *et al.*, 2004). It was shown that 2 mM of 2,3-diCB was less toxic to cells than similar

concentrations of biphenyl or 2-monoCB, and that toxic effects is determined by the solubility and Log K_{ow} (water-octanal partition coefficient; i.e. the likelihood that a compound would partition into an aqueous or organic phase) of the compounds. In constrast, dihydroxylated product from 2,3-diCB was extremely lethal to cells resulting in lyses of cells and a decrease in cell viability to less than 1 % of the control culture (Camara *et al.*, 2004). Though, most of the products of metabolism of 2,3-diCB, and the products from 2,4'-diCB could not be identified in the present study, the available data are consistent with the observations of Camara *et al.* (2004). Results also suggests that the mechanism of toxicity of 2,3- and 2,4'-diCB to cells of strain IA3-A may be different because cultures with lower concentration of 2,4'-diCB had higher numbers of CFU than cultures with higher concentration of the compound, whereas numbers of CFU in cultures with 2,3-diCB were similar irrespective of the initial concentration of the compound (Fig. 2.3).

Attempts to identify 2- and 4-CBA as transformation products of 2,4'-diCB were not successful, nonetheless culture extracts were shown to contain numerous metabolic intermediates (Fig. 2.5 and Table 2.6). The characteristic yellow colour of culture supernatants indicates that *meta*-cleavage compound (HOPDA) was produced from 2,4'-diCB. At neutral pH, the absorption maximum of the supernatant was 397 nm, which shifted to 342 nm at acidic pH (Fig. 2.7). This is consistent with the shift in the wavelength of maximum absorption of certain HOPDA(s) in the presence of borate (Seah *et al.*, 2000). The accumulation of HOPDA in culture supernatant could account for the inability to detect 2- or 4-CBA in culture extracts, because these compounds would be formed at low concentrated if HOPDA accumulates from 2,4'-diCB (Maltseva *et al.*, 1999; Bedard and Haberl, 1990). One of the transformation product of 2,3-diCB was identified as 2,3-diCBA (Fig. 2.5). This

compound could be formed by oxidation of the non-substituted ring of 2,3-diCB (Seeger *et al.*, 1995). Extracts of a culture with 2,3-diCB also contained several transformation products (Fig. 2.5 and Table 2.5). These data suggests that 2,3- and 2,4'-diCB were only cometabolized by *Hydrogenophaga* sp. IA3-A as reported for several PCB-degrading bacterial strains (Kim and Picardal, 2001; Bedard and Haberl, 1990; Ahmed and Focht, 1973).

Hydrogenophaga sp. IA3-A grew on certain aromatic compounds including catechol and protocatechuic acids (Table 2.7), which are common intermediates of the pathways used for dissimilation of aromatic compounds. Poor growth or lack of growth in the presence of succinate indicates that some of these compounds were inhibitory to growth at the concentration that was tested. The rate of oxidation of many of these substrates by biphenyl-grown cells was relatively low when compared to the rate of the oxidation of (chloro)biphenyl (Table 2.8). This suggests that biphenyl-degrading enzymes in cells of strain IA3-A were more specific for (chloro)biphenyl. The high rate of oxygen uptake in the presence of catechol is not surprising because catechol is a common intermediate of many aromatic compounds (Harwood and Parales, 1996). The relatively low uptake of oxygen in the presence of 4-chlorocatechol and 4-methylcatechol was probably due to non-specific activity of 2,3-dihyroxybiphenyl dioxygenase enzyme (Maeda *et al.*, 1995) or catechol 1,2-dioxygenase enzyme (Dorn and Knackmuss, 1978) in biphenyl-grown cells. It is also likely that some of the compounds were not substrates for biphenyl-degrading enzymes of strain IA3-A, or that the compounds were present at an inhibitory concentration.

Chapter 3

Effect of Low Temperature on the Pattern of Attack of Polychlorinated Biphenyls in Commercial Mixtures and Cometabolic Degradation of Selected Chlorobiphenyls by *Hydrogenophaga* sp. IA3-A

3.1. Introduction

PCBs and other halogenated aromatic compounds have been detected in the atmosphere and biota in the Canadian Arctic (Braune and Simon, 2003; Hung *et al.*, 2001). Two sites in the Canadian Arctic, which were former military radar facilities, have also been shown to contain mixtures of PCBs with concentrations exceeding 50 ppm that is set out in the regulations of the Canadian Environmental Protection Act (Environmental Sciences Group, 1996). Because of the remote locations of these sites, soil transportations costs make ex-situ soil treatments prohibitively expensive (Kuipers *et al.*, 2003). Biotreatment of PCB-contaminated Arctic soils is a focus of recent investigations to determine the feasibility of on-site bioremediation (Kuipers *et al.*, 2003). However, relatively few studies have investigated degradation of PCBs by cold-adapted bacteria (Master and Mohn, 1998; Mohn *et al.*, 1997). Therefore, more studies are needed to investigate PCB degradation by cold-adapted bacterial strains because successful bioremediation of PCB-contaminated sites in cold climates would require cold-adapted microorganisms.

Polychlorinated biphenyl-degrading bacteria have been classified into two groups based on their ability to degrade defined PCB mixtures. The first group of bacteria degrade a broad range of PCBs including congeners with two *ortho*-substituted chlorines, but they had weak activity against congeners with two *para*-substituted chlorines. In contrast, the second group

of bacteria degrade a narrower range of PCBs, but they efficiently degraded congeners with two *para*-substituted chlorines and they were relatively weak against congeners with two *ortho*-substituted chlorines (Mondello *et al.*, 1997; Williams *et al.*, 1997). It is widely accepted that degradation of PCBs in most bacteria is initiated by the attack of a dioxygenase at carbon positions 2,3 or 5,6 (Masse *et al.*, 1984; Ahmed and Focht, 1973; Furukawa *et al.*, 1979b). In this regard, the attack of PCBs by most bacteria is limited to congeners having a 2,3 or 5,6 site that is free of chlorines. Therefore, the attack of congeners that are substituted at positions 2,3 or 5,6 could be used to indicate strains that are capable of attacking congeners at carbon positions 3,4 or 4,5 since this group of bacteria are expected to possess a dioxygenase that is uncommon in PCB-degrading strains (Bedard *et al.*, 1987a; Bedard *et al.*, 1986). In addition, some PCB-degrading bacteria convert certain congeners having a ring that is substituted at position 4 to excess level of HOPDA (Bedard and Haberl, 1990).

In order to determine the extents of degradation of PCBs and the chlorination pattern of PCBs that are degraded by *Hydrogenophaga* sp. IA3-A at low and moderate temperatures, Aroclor 1221 containing monoCB- to triCBs, Aroclor 1232 containing monoCBs to hexaCBs, and Aroclor 1248 containing diCB- to hexaCBs were chosen for these purposes. This would allow a comparison of the degradability of the same congeners in mixtures of PCBs having different composition. This chapter reports that biphenyl-grown cells of *Hydrogenophaga* sp. IA3-A are able to cometabolize PCB congeners in commercial mixtures at low temperature. It is believed that this is the first report of PCB degradation at low temperature by a cold-adapted strain belonging in the genus *Hydrogenophaga*. The effect of low temperature on the pattern of attack of PCBs in different commercial (Aroclor) mixtures is described. The degradation of some selected congeners at low temperature with subsequent

production of metabolic intermediates is also the first report of transformation of PCBs to intermediates of the upper-pathway at low temperature.

3.2 Materials and Methods

3.2.1 ***Source and storage of microorganism.*** Cold-adapted biphenyl-degrading strain tentatively identified as *Hydrogenophaga* sp. IA3-A was isolated following the protocols outlined in Chapter 2 (section 2.2.1). Biphenyl-degrading enzymes were induced in cells of strain IA3-A by growing cells in MSM containing 0.5 % (w/v) of biphenyl by using the procedures described in Chapter 2 (section 2.2.2). Biphenyl-grown cells that were suspended in 0.05 M sodium phosphate buffer (pH 7.5) were transferred into 20 % glycerol and stored at -20 °C to serve as stock culture.

3.2.2 ***Growth conditions.*** The MSM used here is the same as that described in section 2.2.2. in chapter 2. Benzoic- or biphenyl-grown cells were suspended in 250-mL flasks containing 100 mL of MSM and 2 mM benzoic acid, 2 mM 2-CBA, or 2 mM 4-CBA to give an initial $OD_{600\ nm}$ of 0.1. Cultures in control flasks contained live cells and 100 mL of MSM only. The cultures were incubated at 30 °C at 200 rpm for 7 days and growth was determined at regular intervals by measuring the $OD_{600\ nm}$.

3.2.3 ***Cometabolism of PCBs.*** Two milliliters of biphenyl-grown cell suspension having a final $OD_{600\ nm}$ of 2.0 was dispensed into 25-mL, acid-washed sterile glass tubes. Cells in control cultures were inactivated with two drops of 70 % perchloric acid. Then, acetone solution of Aroclor 1221, 1232, or 1248 (Crescent Chemical Co., NY, USA) was added into experimental and acid-inactivated control cell suspension to a final concentration of 10 ppm

in order to avoid possible toxicity of the compounds at higher concentration. Thereafter, non-degradable internal standard, 2,2',4,4',6,6'-hexaCB (Ultra Scientific, RI, USA), was added into each tube to a final concentration of 1 or 4 ppm and the reaction were incubated in triplicates at 5 or 30 °C on a shaker at 200 rpm for 48 h. In order to determine the extents of degradation of selected chlorinated biphenyls, 4 mL of biphenyl-grown cell suspension having an $OD_{600\,nm}$ of 1.0 was dispensed into 25-mL acid-washed sterile glass tubes. Cells in control reaction were inactivated with two drops of 70 % perchloric acid. Thereafter, acetone solution of 2,2'-, 2,3-, 2,4'-, 2,6-, 4,4'-diCB or 2,4,4'-triCB (ULTRA Scientific, RI, USA) was added separately into the cell suspension to achieve a final diCB and triCB concentration of 500 μM and 100 μM, respectively. Each of the cell suspension also contained approximately 3 μM of 2,2',4,4',6,6'-hexaCB. All the tubes were incubated in triplicates at 5 or 30 °C on a shaker at 200 rpm for 72 h. Reactions were stopped at the end of incubation period with two drops of 70 % perchloric acid or by freezing the samples followed by storage at -20 °C.

3.2.4 ***Preparation of cell-free extracts.*** Two grams of washed cells were suspended in 4 ml of 50 mM 2-(*N*-morpholino)ethanesulfonic acid (MES) buffer (pH 6.0) containing 1 mM EDTA (ethylenediaminetetracetic acid), 1 mM dithiothreitol, and 15 % (vol/vol) glycerol. The suspension was sonicated (Braunsonic 2000, Canlab) with constant cooling for 6 min with intervals of 30 s to cool the probe. Then, the suspension was centrifuged at 45, 000 *g* for 1 h at 4 °C using an Optima L-90 K ultracentrifuge (Beckman Coulter Inc., CA, USA). The supernatant formed the source of extracts for enzyme assay.

3.2.5 ***Analytical methods.*** Biphenyl dioxygenase was assayed spectrophotometrically at 30 °C by measuring the decrease in absorbance of NADPH at 340 nm due to the presence of extracts of cells grown on either biphenyl (0.5 %, w/v), TSB (500 mL), succinate (10 mM) or succinate (10 mM) plus 2,4'-diCB (44.8 μM). A total of 1 mL reaction mixture contained 250 μM NADPH, 1.09 μM FAD, 500 μM $Fe(NH_4)_2(SO_4)_2$, 1 mM biphenyl or 2,4'-diCB, and 100 µL of cell extract. The reaction was initiated by adding biphenyl or 2,4'-diCB to the reaction mixtures. The decrease in absorbance was measured against a blank mixture that contained no substrate. Control mixtures were included to measure endogenous oxidation of NADPH. Spectrophotometric studies were done using a Shimadzu UV-260 spectrophotometer. Specific activity was defined as nmol of NADPH oxidized per min per milligram of protein.

Oxygen consumption by whole cells, grown on 4 mM benzoic or 0.5 % (w/v) of biphenyl, in the presence different chlorobenzoates or chlorinated biphenyls was determined using a Clark-type oxygen electrode following the procedures outlined in chapter 2 (section 2.2.5). Protein concentration was determined by the method of Bradford (1976) with bovine serum albumin as a standard.

3.2.6 ***Extraction and analysis of metabolic intermediates.*** Duplicate or triplicate samples were centrifuged (15, 000 *g*, 4 °C, 15 min) to remove cells using a Sorvall RC-5 centrifuge. The supernatant (4 mL) was extracted with two volumes of hexane at neutral pH with agitation at 250 rpm for 24 h. Thereafter, the aqueous phase was collected and acidified with 1 M HCl to pH 3, and polar metabolites were extracted with two volumes of ethyl acetate by agitating at 250 rpm for 24 h. The organic phase was dried over anhydrous sodium sulfate,

and the extracts were evaporated under a gentle stream of nitrogen gas. The residues were derivatized according to the method of Maltseva *et al.* (1999) with slight modification using 20 μL of pyridine and 80 μL of N-O-bis-(trimethylsilyl)-trifluoroacetamide containing 1% trimethylchlorosilane (TMS) followed by incubation at 60 °C for 30 min. GC-MS was performed with a Varian CP-3800 gas chromatography connected to a Varian Saturn 2000 ion trap mass spectrometer. The GC was equipped with a ZB-5 capillary column with 5 % phenyl polysiloxane (30 m by 0.25 mm [inner diameter] and 0.25 μm film thickness). The system was operated in spitless mode with a head pressure of 10 psi. The volume of samples injected was 1 μL, and the carrier gas was helium at a flow rate of 1 mL min^{-1}. The temperature of the injector and the transfer line was 260 and 290 °C, respectively. The column temperature was increased from 140 °C to 320 °C at the rate of 5 °C min^{-1}. Where applicable, CBAs were identified by matching the GC retention times and mass spectra of trimethylsilyl-derivatives with authentic trimethylsilated standards of CBAs, and they were quantified using calibration curves prepared for each isomer.

The formation of ring fission products from chlorinated biphenyls and PCB mixtures was monitored spectrophotometrically in supernatants at 397 nm and 430 nm, which are characteristic absorption maxima of the *para*- and *ortho*-HOPDAs, respectively (Gilbert and Crowley, 1997; Seegar *et al.*, 1995).

3.2.7 ***Extraction and analysis of PCBs.*** Samples were thawed and one-tenth volume of 10 % (v/v) Triton X-100 was added into each sample to enhance desorption of PCBs from cells and glass-ware (Bedard *et al.*, 1986). The tubes were shaken on a vortex to enhance

desorption of residual PCBs from cells and glass ware. Then, two volume of hexane was added into each mixture, followed by the addition of anhydrous sodium sulfate (80 mg mL^{-1}) to prevent the formation of a stable emulsion. Prior to extraction, the caps of the tubes were lined with aluminium foil and samples were extracted for 24 h at 250 rpm on a rotary shaker. Thereafter, the extracts were transferred into vials for GC-MS analysis.

Chlorobiphenyls and PCBs extracts were analyzed using the Varian CP-3800 GC and Varian Saturn 2000 ion trap mass spectrometer that are described above under similar conditions. Alternatively, some extracts were analyzed using an Agilent 6890N GC (Agilent Technologies Inc., CA, USA) coupled with a HP-5MS capillary column (30 m x 0.25 mm x 0.25 μm; Hewlett-Packard), and connected to an Agilent 5973 inert mass selective detector. The system was operated in selective ion monitoring (SIM; i.e. selected ions of interest were monitored) mode and the nominal head pressure was 10.75 psi. The carrier gas was helium at a flow rate of 1 mL min^{-1}. The temperature of the injector and the transfer line was 260 and 280 °C, respectively. The samples, 1 μL for extracts analyzed with the Varian GC system were injected manually or 2 μL for extracts analyzed with the Agilent system were injected by an autoinjector (model 7683, Agilent Technologies). For analysis of extracts containing single congeners, the column temperature was held at 140 °C for 1 min, then increased at 10 °C min^{-1} to 230 °C and held for 10 min. The extracts containing PCBs mixtures in Aroclor 1221 or 1232 were analyzed using the method of Mohn *et al.* (1997). The column temperature was held at 104 °C for 3 min, and then increased to 160 °C at a rate of 20 °C min^{-1}, and increased at 2.5 °C min^{-1} to 233 °C, and increased to 290 °C at 20 °C min^{-1}, and held for 3 min. The extracts containing PCBs in Aroclor 1248 were analyzed using the temperature program of Bedard *et al.* (1986). The temperature program was held at 40 °C for

2 min, then raised to 80 °C at 10 °C min^{-1}, then to 225 °C at 6 °C min^{-1}, and held at 225 °C for 10 min. The ions monitored for monoCBs, diCBs, triCBs, tetraCBs, pentaCBs, and hexaCBs were *m/z* 188 to 190, *m/z* 222 to 226, *m/z* 256 to 262, *m/z* 290 to 292, *m/z* 326 to 328, and *m/z* 358 to 370, respectively. The assignment of congeners in the extracts containing commercial mixtures of PCBs were done by comparing retention times of each peak to published data (Mohn *et al.*, 1997; Frame *et al.*, 1996; Mullin *et al.*, 1984; Erickson *et al.*, 1982; Albro *et al.*, 1977). The peaks of single congeners and PCB congeners in commercial mixtures were quantified by normalizing the area of individual peak in the experimental and control to the area of the peak corresponding to the internal standard, which allowed the estimation of the percentage degradation of each congener. Biological removal of each congener was estimated as the difference between the amounts of that congener in live cultures compared to the amounts in control cultures.

3.3 Results

3.3.1 ***Cometabolism of PCBs congeners in Aroclor 1221.*** PCB congeners in Aroclor 1221 that was incubated with biphenyl-grown cells for 48 h at 5 or 30 °C were degraded to different extents (Fig. 3.1 and 3.2). The pattern and extents of degradation of the congeners were different at 5 ° and 30 °C (Fig. 3.3). Quantification of the extent of degradation of PCBs showed that 14 or 16 of the congeners were partly or significantly removed in cell suspensions incubated at 5 ° or 30 °C, respectively. The retention times of the congeners in Aroclor 1221 are shown in Table 3.1.

All the monoCBs (peaks 2, 3, 4), 2,4- and 2,5-diCB (peak 6) were removed in cell suspension incubated at 30 °C. (Fig. 3.3). There was considerable removal of biphenyl, 2,3'-, 3,4- and 3,4'-diCB. While 2,3- and 2,4'- (peak 8), and 4,4'-diCB (peak 14) were partly removed. There was little or no removal of 2,2'- and 2,6- (peak 5), as well as 3,3'-diCB. For the triCBs, there was considerable removal of 2,4,4'- and 2,4',5- (peak 20), 2,3,3'- and 2'3,4- (peak 21), 2,3,4'- (peak 22), and 3,4,4'-triCB (peak 23), but 2,2',4- (peak 13), 2,4',6- (peak 17), and 2,3',4- triCB (peak 19) were only partly removed. There was little or no removal of 2,2',5- (peak 12), 2,3,6- and 2,3',6- (peak 15), 2,2',3- (peak 16), and 2,3',5-triCB (peak 18). At 30 °C, congeners with single chlorine atom on one ring were completely removed. As for the diCBs, only 2,4- and 2,5-diCB (peak 6), congeners having two chlorines on a single ring and a free phenyl ring were completely removed. The other diCBs, which have two chlorines on one ring or a chlorine atom on each ring, were less preferentially attacked or they were not attacked at all. All the triCBs, which have three chlorines on a single ring or at least a chlorine substituent on one of the two rings were not removed or were less preferentially

attacked. The extents of removal of some triCBs were not largely different from the extents of degradation of many of the diCBs.

The pattern of removal of PCB congeners in Aroclor 1221 in cell suspension incubated at 5 °C for 48 h was largerly different when compared to the pattern of removal of PCB congeners in cell suspension incubated at 30 °C. In addition, none of the congeners in cell suspension incubated at 5 °C was totally removed (Fig. 3.3). However, all the monoCBs (peaks 2, 3, 4), 2,4- and 2,5- (peak 6), 2,3'- (peak 7), 2,3- and 2,4'- (peak 8), 3,4- and 3,4'-diCB (peak 11), 2,3',5- (peak 18), and 2,3',4-triCB (peak 19) were considerably removed, while 3,3'- (peak 10), 4,4'-diCB (peak 14), 2,2',3- (peak 16), and 2,3,4'-triCB (peak 22) were partly removed. There was no removal or poor removal of 2,2'- and 2,6-diCB (peak 5), 2,2',3- (peak 16), 2,2',4- (peak 13), 2,2',5- (peak 12), 2,2',6- (peak 9), 2,3,3'- and 2',3,4- (peak 21), 2,4',6- (peak 17), and 3,4,4'-triCB (peak 23). Surprisingly, removal of biphenyl, 2,3- and 2,4'-diCB (peak 8), 2,3',5- (peak 18), 2,3',4- (peak 19), and 2,4,4'- and 2,4',5-triCB (peak 20) was slightly higher in cell suspensions incubated at 5 °C when compared to cell suspensions incubated at 30 °C. In addition, few of the congeners including 3,3'-diCB (peak 10), 2,3,6- and 2,3',6-triCB (peak 15) that were not removed at 30 °C were partly removed at 5 °C.

3.3.2 *Cometabolism of PCB congeners in Aroclor 1232 and Aroclor 1248*

Forty-six peaks corresponding to monoCBs, diCBs, triCBs, tetraCBs, pentaCBs, and hexCBs were resolved by GC-MS analysis of extracts of cell suspensions containing Aroclor 1232 (Table 3.2; see appendix A). However, some of the peaks contained congeners with

similar retention times. The number of congeners that were removed from Aroclor 1232 in cell suspensions incubated at 5 °C was 22, while the number of congeners removed in cell suspensions incubated at 30 °C was 30. The congeners removed at both temperatures included monoCB, diCBs, triCBs, and a tetraCB congener. However, none of the congeners corresponding to pentaCB or hexaCB was removed at 5 ° or 30 °C. At 5 °C, the extent of removal of PCBs was between 34 to 100 % (Fig 3.4 and Fig. 3.6), while the extent of removal of PCBs at 30 °C was between 18 to 100 % (Fig. 3.5 and Fig. 3.6).

After 48 h, the extents of removal of monoCBs were similar in cell suspensions incubated at 5 ° and 30 °C. The profile and extents of removal of diCBs (peaks 4, 5, 6, 7, 9, 10, 11) at 5 ° and 30 °C were also similar, except for 2,2'- and 2,6- (peak 4), and 4,4'-diCB (peak 11) that were not removed at 5 °C (Fig. 3.6). Unlike in cell suspensions incubated at 5 °C, all the triCBs (peaks 11 – 19) present in Aroclor 1232 were removed to different extents in cell suspensions incubated at 30 °C. The extents of removal of many of the triCBs, including 2,3,2'- and 2,6,4'- (peak 14), 2,5,3'- and 2,4,3'- (peak 15), 2,4,4'- and 2,5,4'- (peak 16), 3,4,2'- (peak 18), and 2,3,4'-triCB (peak 19), at 5 ° and 30 °C were generally similar. TriCBs like 2,5,2'- (peak 11), 2,4,2'- (peak 12), 2,3,6- and 2,3,6'- (peak 13), and 2,3,3'- (peak 17) were resistant to removal at 5 °C. With the exception of 2,4,5,2'-tetraCB (peak 25), none of the tetraCBs was removed at 5 ° or 30 °C. The extent of removal of 2,4,5,2'-TeCB (peak 25) at 30 °C was considerably higher than the extent of removal of this congener at 5 °C (Fig. 3.6). Although there was considerable removal of congeners corresponding to 3,4,2'-triCB and 2,5,2',6'-tetraCB (peak 18), and 2,3,4'-triCB and 2,4,2',6'-tetraCB (peak 19), however, comparison of the relative abundance of the respective congeners in Aroclor 1232 (Table 3.2) indicates that it is likely that the difference in the area of the peaks

corresponding to these congeners prior to incubation and the area of the peaks after incubation was due to removal of 3,4,2'- and 2,3,4'-triCB, and not 2,5,2',6'- or 2,4,2',6'-tetraCB, respectively.

After 48 h, only 5 and 14 of the 32 PCB congeners that were resolved by GC-MS were removed by cells incubated with Arolcor 1248 at 5 ° and 30 °C, respectively (Table 3.3). PCBs that were removed at 30 °C include 2,4- and 2,5- (peak 1), 2,3'-diCB (peak 2), 2,2',5- (peak 3), 2,2',4- (peak 4), 2,2',3- and 2,4',6- (peak 5), 2,3',4- (peak 6), 2,4',5- (peak 7), 2,4,4'- (peak 8), 2',3,4-triCB (peak 9), 2,2',3,4-, 2,3,4',6- and 2,3',4',6-TetraCB (peak 18). At 5 °C, only 2,3'-diCB (peak 2), 2,2',4- (peak 4), 2,2',3- and 2,4',6- (peak 5), and 2,4,4'-triCB (peak 8) were removed. Among the tetraCBs, the congener that was considerably removed at 30 °C was more likely to be 2,3,4',6-tetraCB (from peak 18), because this congener has a free 2,3- position (Table 3.3) on the *para*-substituted ring that was open to dioxygenation.

3.3.3 ***Cometabolism of selected chlorobiphenyls.*** Cell suspension of strain IA3-A incubated at 5 ° or 30 °C efficiently removed 2,4'-diCB and 2,4,4'-triCB. At 5 ° and 30 °C, 342 ± 21 and 414 ± 8 μM of 2,4'-diCB was removed, respectively. Only 173.0 ± 17.0 and 221.0 ± 13.0 μM of 2,3-diCB was removed at 5 ° and 30 °C, respectively. At 5 °C, 4,4'-diCB remaining in the experimental and control was similar, and only 34 ± 7 % of 4,4'-diCB was removed in cell suspension incubated at 30 °C. Percentage removal of 2,4,4'-triCB at 30 °C after 72 h was 90 ± 3 %, while percentage removal of the congener at 5 °C after 72 h was 75 ± 3 %. There was no obvious difference in the amount of 2,2'- or 2,6-diCB in live cell

suspension and acid-inactivated cell suspension incubated at 5 ° or 30 °C, although low level of *ortho*-chlorinated HOPDA was formed from 2,2'-diCB at 30 °C, indicating that the congener was degraded, albeit to minimal extent.

3.3.4 ***Detection of metabolic intermediates.*** *Ortho* and *para*-HOPDAs were formed from many of the PCB mixtures. There were higher concentrations of *ortho*-HOPDAs from all the Aroclor mixtures when compared to the concentrations of *para*-HOPDAs (Fig. 3.7). In addition, the accumulation of *ortho*- or *para*-HOPDAs from PCBs in Aroclor 1221 was higher than accumulation of the compounds from PCBs in Aroclor 1232, 1248, and 1254. The extents of accumulation of *ortho*- or *para*-HOPDAs from PCBs in Aroclor 1232 and Aroclor 1248 were similar. The compounds were not formed from Aroclor 1254 after 24 h of incubation.

The major trimethysilated intermediate that was detected in the extracts of supernatants of cell suspension incubated with 2,3'-diCB was similar to trimethylsilated standard of 2,3-diCBA. All the 2,3-diCB that was degraded at 5 or 30 °C was recovered as 2,3-diCBA. The CBAs formed from 2,4'-diCB at 5 or 30 °C were identified as 2- and 4-CBA (Fig. 3.8 and Fig. 3.9). At 30 °C, only 30 and 16 % of the 2,4'-diCB that was degraded was recovered as 2- and 4-CBA, while 21 and 5 % of the degraded congener was recovered as 2- and 4-CBA at 5 °C, respectively. Spectral scanning of supernatants of cell suspension incubated with 2,3-diCB showed that HOPDA did not accumulate in the broth. In contrast, HOPDA appear to be the major metabolite produced from 2,4'-diCB (Fig. 3.10). Low level of accumulation of HOPDA in supernatants of cell suspension incubated with 2,2'-diCB (Fig. 3.10) at 30 °C

supports previous conclusion that the congener was resistant to degradation. Chlorinated-HOPDA was not detected in the supernatant of cell suspension incubated with 2,6-diCB.

3.3.5 ***Growth and oxygen uptake in the presence of CBAs and CBs.*** Biphenyl or benzoate-grown cells could grow on benzoate but cells failed to grow on 2- or 4-CBA after seven days of incubation. The rate of oxidation of biphenyl was higher than the rate of oxidation of benzoate or 2,4'-diCB by biphenyl-grown cells (Table 3.4). Biphenyl-grown cells oxidized 4,4'-diCB or 2,4,4'-triCB at a relatively lower rate, and the rate of oxidation of 2,2'-diCB was much lower. Biphenyl-grown cells did not oxidize 2,6-diCB. Likewise, none of the CBAs was oxidized by biphenyl-grown cells. The rate of oxidation of biphenyl or benzoate by benzoate-grown cells was only moderate, and the rate of oxidation of 2-, 4-, 2,3-CBA, and 2,4'-diCB by these cells was much lower. Benzoate-grown cells failed to oxidize 2,6-CBA.

3.3.6 ***Enzymatic studies.*** Extracts of cells grown on biphenyl, succinate, succinate plus 2,4'-diCB, or TSB contained the activity of biphenyl dioxygenase (Table 3.5). Though 2,4'-diCB was not a growth substrate for strain IA3-A, activity of the enzyme was higher in extracts of cells grown on succinate with low concentration of 2,4'-diCB than in extracts of cells grown on succinate only, and biphenyl dioxygenase was induced to a much higher level by growth on biphenyl. There was relatively moderate level of constitutive enzyme production after growth on TSB, while constitutive production of the enzyme was much lower by growth on succinate only.

Table 3.1. Relative retention times and percent composition of biphenyl and PCB congeners in Aroclor 1221

Peak no.	Relative retention time[a]	Congener assignment(s)	Amount (weight %)[b]
1	0.308	Biphenyl	19
2	0.375	2	27.25
3	0.424	3	2.85
4	0.430	4	15.07
5	0.462	2,2'	5.51
5	0.462	2,6	0.49
6	0.503	2,4	1.45
6	0.503	2,5	1.38
7	0.521	2,3'	2.91
8	0.531	2,3	0.72
8	0.531	2,4'	9.81
9	0.548	2,2',6	0.11
10	0.595	3,3'	0.09
11	0.607	3,4	0.6
11	0.607	3,4'	1.07
12	0.611	2,2',5	0.76
13	0.614	2,2',4	0.38
14	0.619	4,4'	3.87
15	0.628	2,3,6	0.04
15	0.628	2,3',6	0.15
16	0.650	2,2',3	0.34
17	0.655	2,4',6	0.17
18	0.696	2,3',5	0.15
19	0.701	2,3',4	0.12
20	0.717	2,4,4'	0.61
20	0.717	2,4',5	0.55
21	0.737	2,3,3'	0.07
21	0.737	2',3,4	0.50
22	0.756	2,3,4'	0.25
23	0.864	3,4,4'	0.18

[a]Retention times of congeners (in a ZB- capillary column with 5 % phenyl polysiloxane) are relative to that of the internal standard, 2,2',4,4',6,6'-hexaCB, which had a retention time of 22.7 min.

[b]Amount (weight %) of PCB congeners was adopted from Mohn *et al.* (1997), and is only an estimation since the percent composition of PCBs varies among lots of Aroclors.

Table 3.2. Retention times and percent composition of PCB congeners in Aroclor 1232

Peak no.	Retention time (min.)	Congener[a]	Amount[b] (weight %)
1	8.12	2	15.21
2	9.05	3[c]	1.98
3	9.24	4[c]	10.36
4	9.92	**2,2'**/2,6	**5.32**/0.60
5	10.74	2,4/2,5	1.12/1.25
6	11.13	2,3'	3.02
7	11.36	2,3/**2,4'**	0.49/**10.71**
8	12.17	2,6,2'	0.46
9	12.68	3,5	0.02
10	12.94	3,4/**3,4'**	0.35/**0.73**
11	13.18	4,4'/**2,5,2'**	3.24/**4.89**
12	13.58	2,4,2'	1.83
13	13.94	2,3,6/2,6,3'	0.08/0.12
14	14.54	**2,3,2'**/2,6,4'	**1.79**/1.08
15	14.78	**2,5,3'**/2,4,3'	**0.75**/0.37
16	14.88	2,4,4'/**2,5,4'**	3.92/**4.17**
17	15.21	2,3,3'	0.42
18	15.71	**3,4,2'**/2,5,2',6'	**2.84**/0.37
19	16.11	**2,3,4'**/2,4,2',6'	**1.62**/0.12
20	16.39	2,3,6,2'	0.47
21	16.78	2,3,2',6'	0.19
22	17.01	2,5,2',5'	1.83
23	17.25	2,4,2',5'	1.37
24	17.42	2,4,2',4'	0.49
25	17.87	2,4,5,2'	0.61

Table 3.2 (Cont'd)

Peak no.	Retention time (min.)	Congener[a]	Amount[b] (weight %)
26	18.11	2,4,6,4'	0.02
27	18.28	**3,4,4'**/2,3,2',4'	1.81
28	18.77	2,3,2',5'	**1.15**/0.66
29	19.24	2,3,4,2'/**2,3,6,4'**/2,6,3',4'	0.36/**0.87**/0.54
30	19.55	2,3,2',3'	0.40
31	19.87	2,4,5,4'	0.92
32	20.10	2,5,3',4'	1.90
33	20.33	**2,4,3',4'**/2,3,6,2',5'	**1.74**/0.30
34	20.52	**2,3,3',4'**/2,3,4,4'	**0.93**/0.61
35	20.96	**2,3,6,2',3'**/2,3,5,2',5'	**0.20**/0.05
36	21.79	2,4,5,2',5'	0.33
37	21.93	2,4,5,2',4'	0.21
38	22.24	2,3,5,2',3'	0.05
39	23.50	2,4,5,2',3'	0.18
40	23.80	2,3,4,2',5'	0.22
41	24.14	2,3,4,2',4'	0.17
42	25.46	3,4,3',4'/**2,3,6,3',4'**	0.17/**0.38**
43	25.75	2,3,4,2',3'	0.12
44	27.16	2,3,5,6,2',5'	0.01
45	27.38	2,3,5,3',4'	0.03
46	28.96	**2,4,5,3',4'**/2,3,6,2',4',5'	**0.29**/0.05

[a]Boldface indicates major congener in the peak (See appendix A for mass spectral identification of peaks).

[b]Percent composition of PCB congeners were adapted from Frame *et al.* (1996).

[c]Congeners in peak no. 2 and 3 in the extracts of control cultures were integrated together due to a much similar retention times, however, both peaks were absent in extracts of experimental cultures incubated at 5 ° or 30 °C for 48 h.

Table 3.3. Cometabolic degradation of some PCB congeners in Aroclor 1248 by biphenyl-grown resting cells of *Hydrogenophaga* sp. IA3-A incubated for 48 h

Peak no.[b]	Congener[c]	Percent degradation[a]	
		5 °C	30 °C
1[d]	2,4; **2,5**	6	19
2	2,3'	72	86
3	2,2',5	0	32
4	2,2',4	39	53
5[d]	2,2',3; **2,4',6**	53	43
6	2,3',4	7	48
7	2,4',5	0	32
8	2,4,4'	58	47
9	2',3,4	15	50
18[d]	2,2',3,4; **2,3,4',6**; 2,3',4',6	0	49

[a]N = 3; and degradation of 15 % or less is not considered to be significant.

[b]Peak no. corresponds to the elution profile of congeners on GC-MS total ion chromatogram.

[c]Aroclor 1248 was analyzed using a Varian GC-MS system and the identities of the congeners was assigned according to previous data (Frame *et al.* [1996]; Bedard *et al.* [1987]; Albro *et al.* [1981])

[d]Peaks containing congeners with similar retention time; congeners in bold are likely to be dominant.

Table 3.4. Oxygen uptake by cells of *Hydogenophaga* sp. IA3-A grown on different substrates

	Growth substrate	
Test substrate[a]	Benzoic	Biphenyl
2-CBA	1.52 ± 0.08	0
4-CBA	1.36 ± 1.44	0
2,3-CBA	2.34 ± 2.81	0
2,6-CBA	0	0
Benzoate	13.05 ± 0.99	11.29 ± 2.61
Biphenyl	41.29 ± 0.97	147.44 ±22.31
2,4'-DiCB	1.52 ± 1.42 (4.59 ± 1.42)	48.12 ± 11.3
2,2'-DiCB	NT	0.35 ± 2.31
2,6-DCB	NT	0
4,4'-DiCB	NT	16.01 ± 13.06
2,4,4'-TriCB	NT	2.84 ± 0.82

Specific activity: nmol of O_2 utilized min^{-1} mg^{-1} protein; N = 3 ± DS, except where stated otherwise; [a]Concentration of test substrates: benzoate and CBAs, 2 mM; Biphenyl, 250 μM; 2,2'-, 2,6-, 4,4'-diCB, 500 μM; 2,4'-diCB, 250 μM or 500 μM for value in parenthesis; 2,4,4'-triCB, 100 μM. NT, not tested.

Table 3.5. Biphenyl dioxygenase activity in extracts of cells of *Hydrogenophaga* sp. IA3-A grown on different carbon sources

Growth substrate or media[b]	Oxidation of NADPH[a] (nmol min^{-1} mg^{-1} protein) in the presence of test substrate	
	Biphenyl[c]	2,4'-DiCB[c]
Biphenyl	12.65	17.2
Succinate	NT	1.22
Succinate + 2,4'-DCB	1.97	5.22
TSB	3.75	5.4

[a]Values are averages of duplicate determination.

[b]Concentration of growth substrates or volume of media: 0.5 % (wt/vol) biphenyl; 10 mM succinate; 10 mM succinate plus 44.82 μM 2,4'-diCB; 500 ml of TSB.

[c]Final concentrations and composition of 1 ml reaction mixtures: 250 μM NADPH; 1.09 μM FAD; 500 μM Fe(NH4)2(SO4)2; 100 µL of cell extract; and 1 mM biphenyl or 2,4'-diCB.

NT, not tested

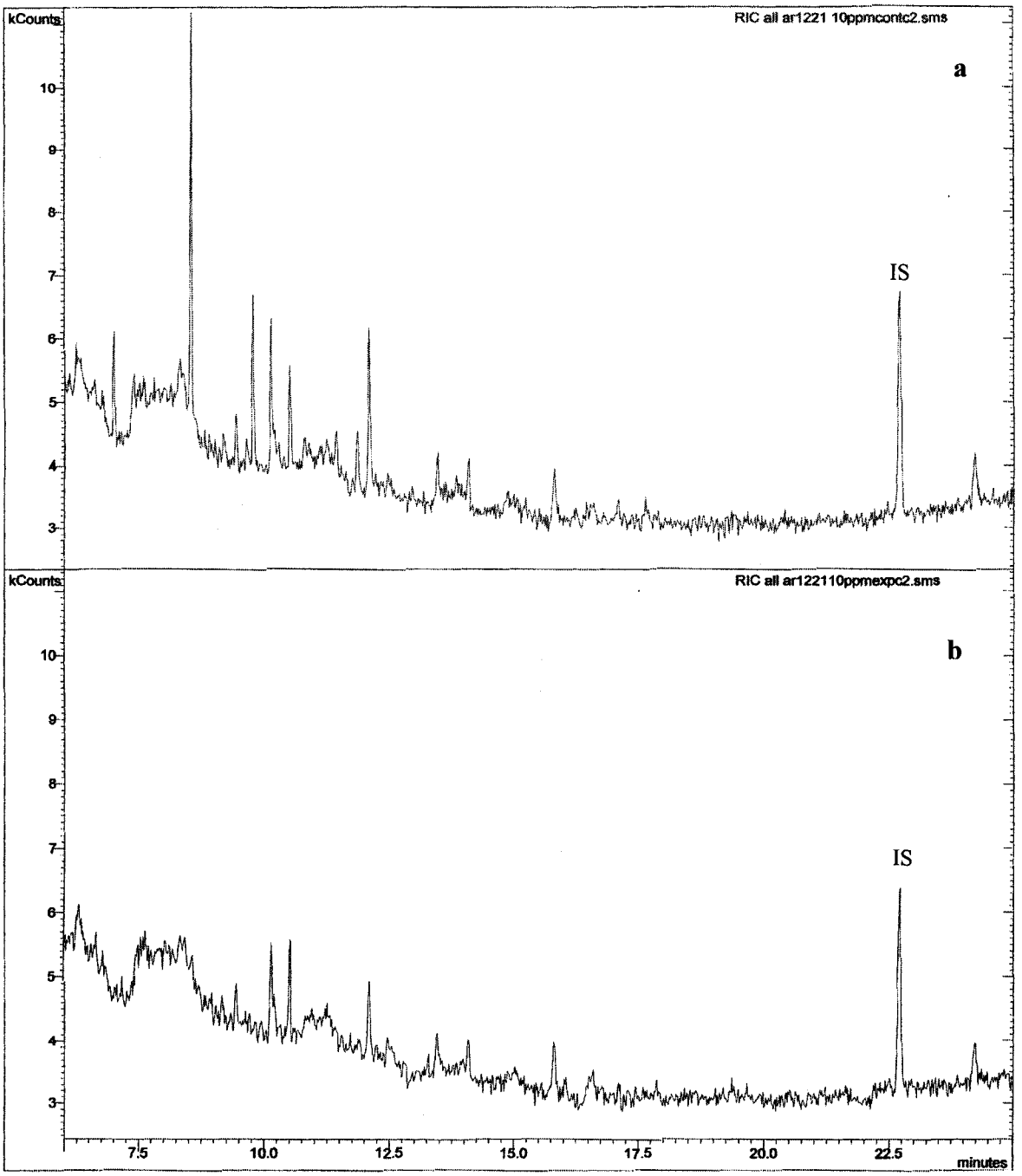


Fig. 3.1. Change in profiles of total ion chromatogram (TIC) of extracts of cultures incubated with Aroclor 1221 at 30 °C for 48 h. a, acid-inactivated cell suspension; b, live cell suspension; IS, internal standard. Extracts were analyzed with the Varian GC-MS system described in materials and methods.

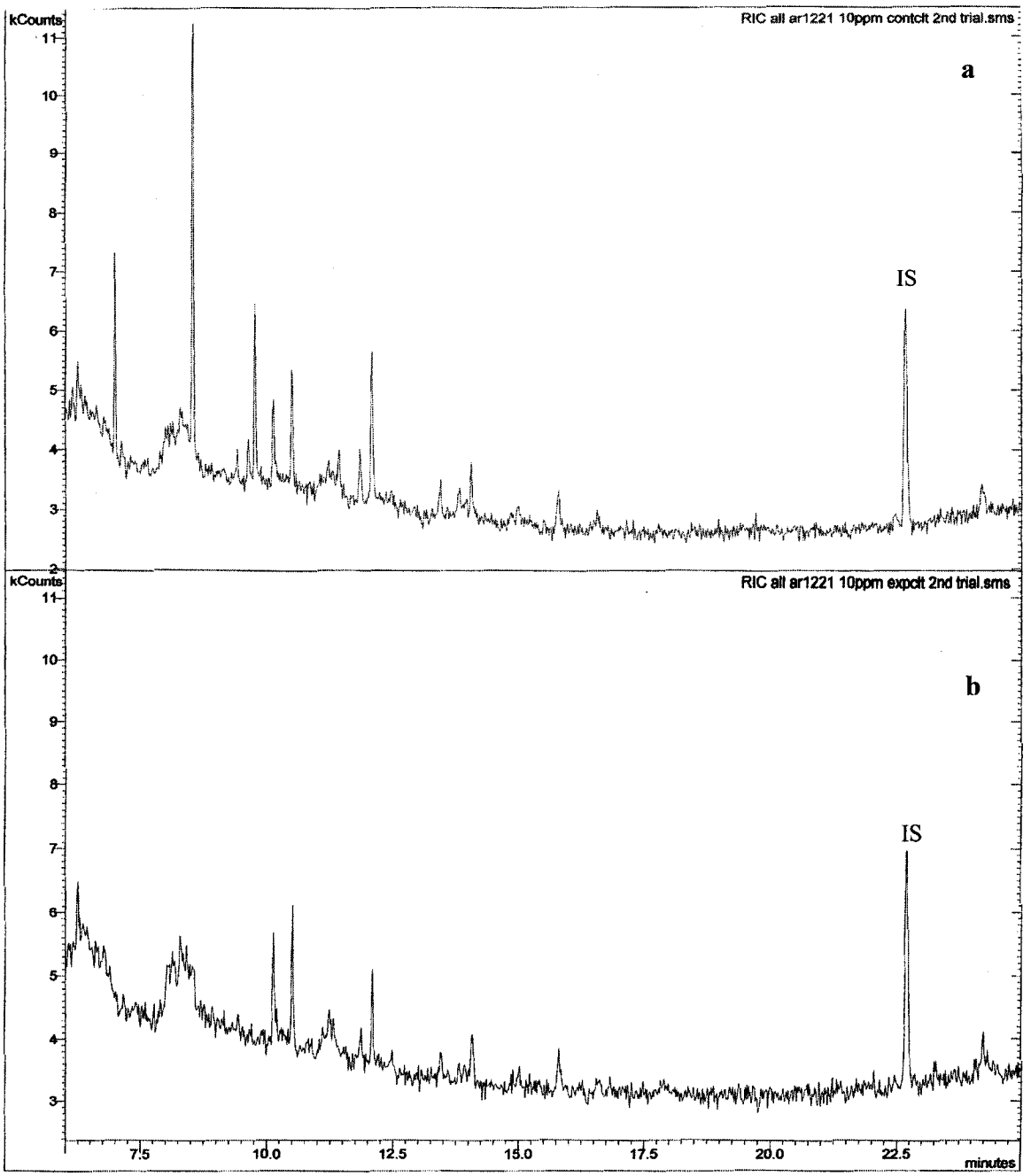


Fig. 3.2. Change in profiles of total ion chromatogram (TIC) of extracts of cultures incubated with Aroclor 1221 at 5 °C for 48 h. a, acid-inactivated cell suspension; b, live cell suspension; IS, internal standard. Extracts were analyzed with the Varian GC-MS system described in materials and methods.

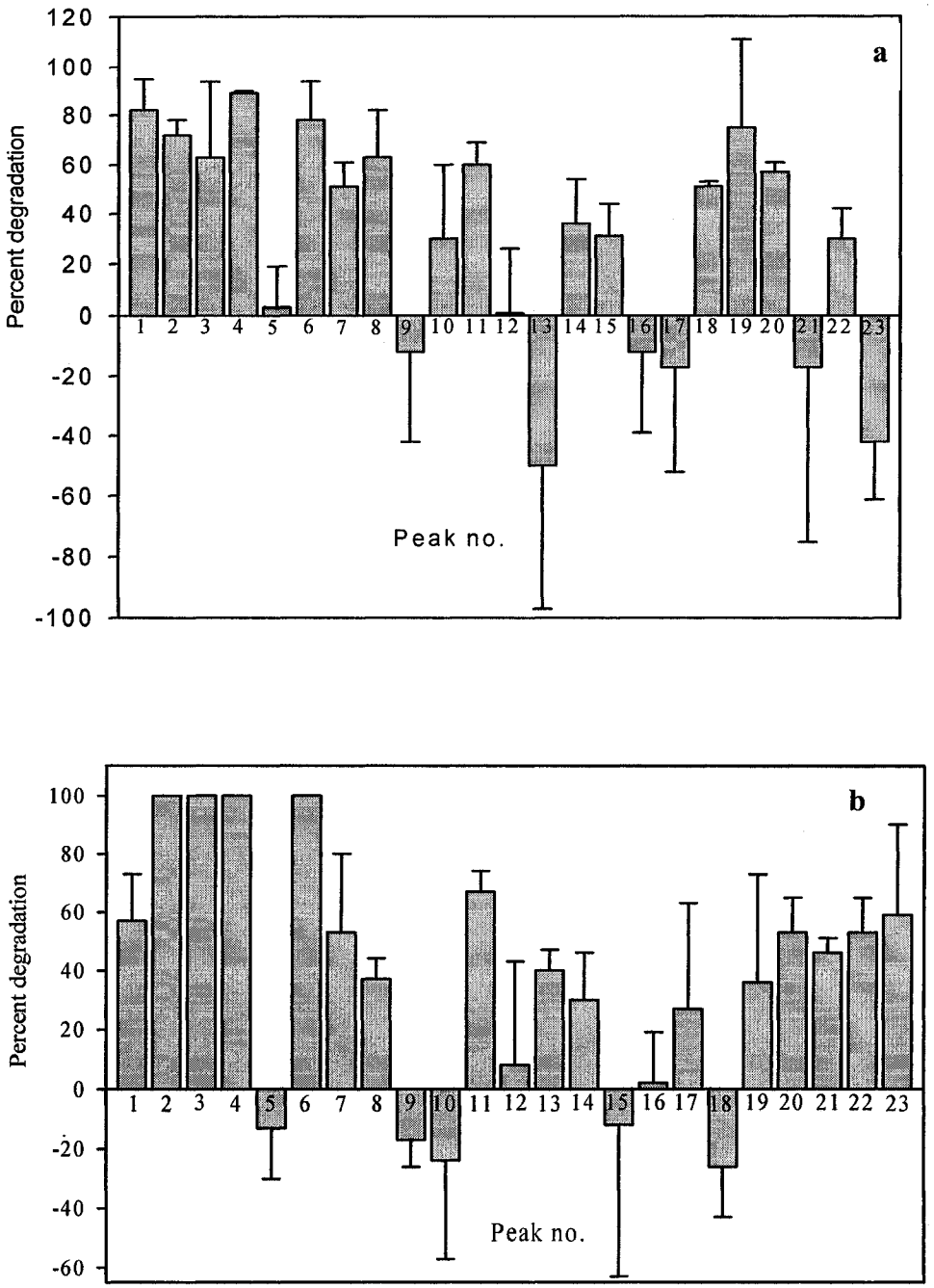


Fig. 3.3. Cometabolic degradation of biphenyl and PCB congeners in Aroclor 1221 by *Hydrogenophaga* sp. IA3-A incubated at 5 ° (a) or 30 °C (b) for 48 h. N = 3; error bars shows standard deviation. Peaks with negative values were poorly recovered in live cell suspension when compared to the respective controls.

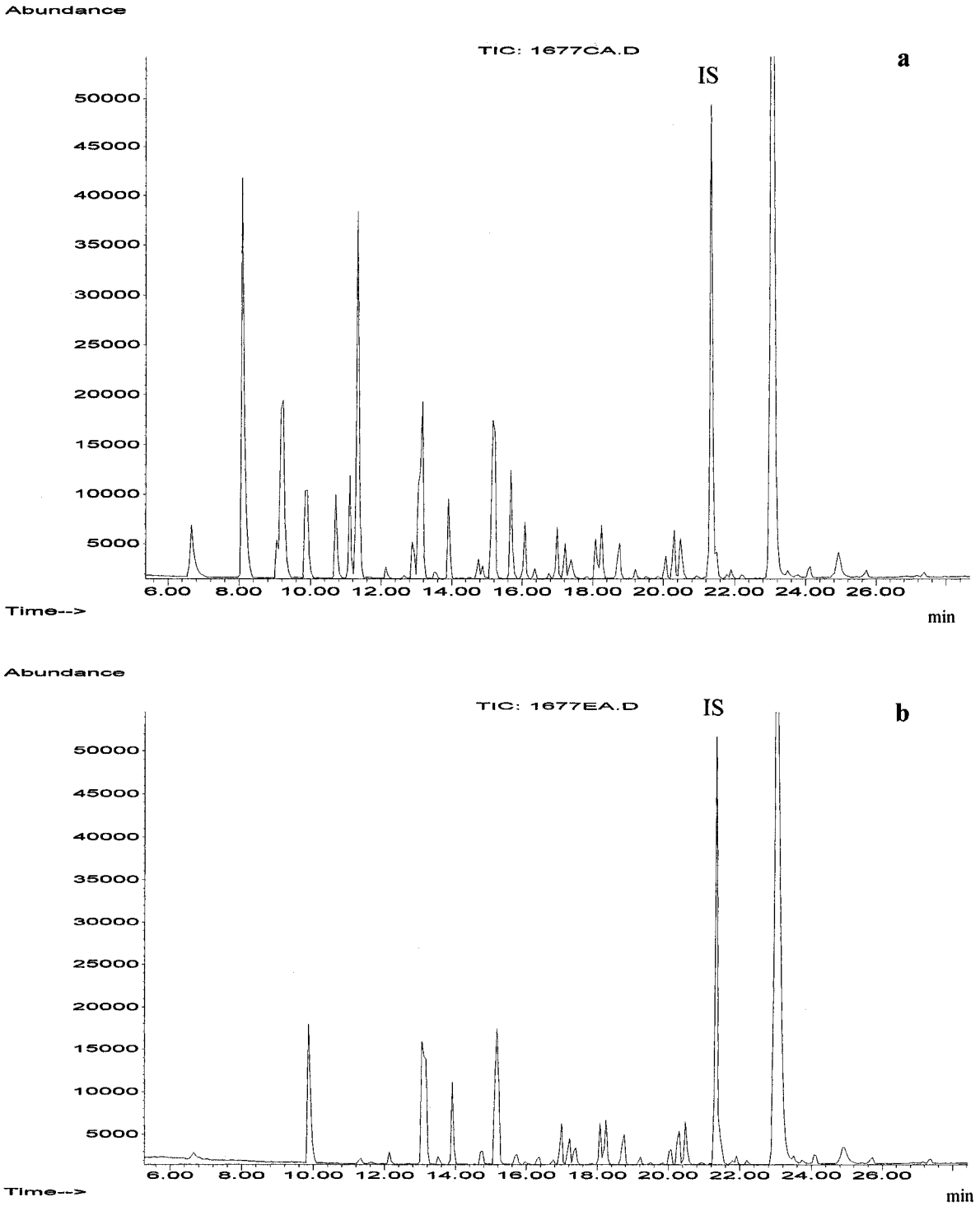


Fig. 3.4. Change in profile of TIC of extracts of cultures incubated with Aroclor 1232 at 5 °C for 48 h. a, acid-inactivated cell suspension; b, live cell suspension. Extracts were analyzed with an Agilent GC-MS system.

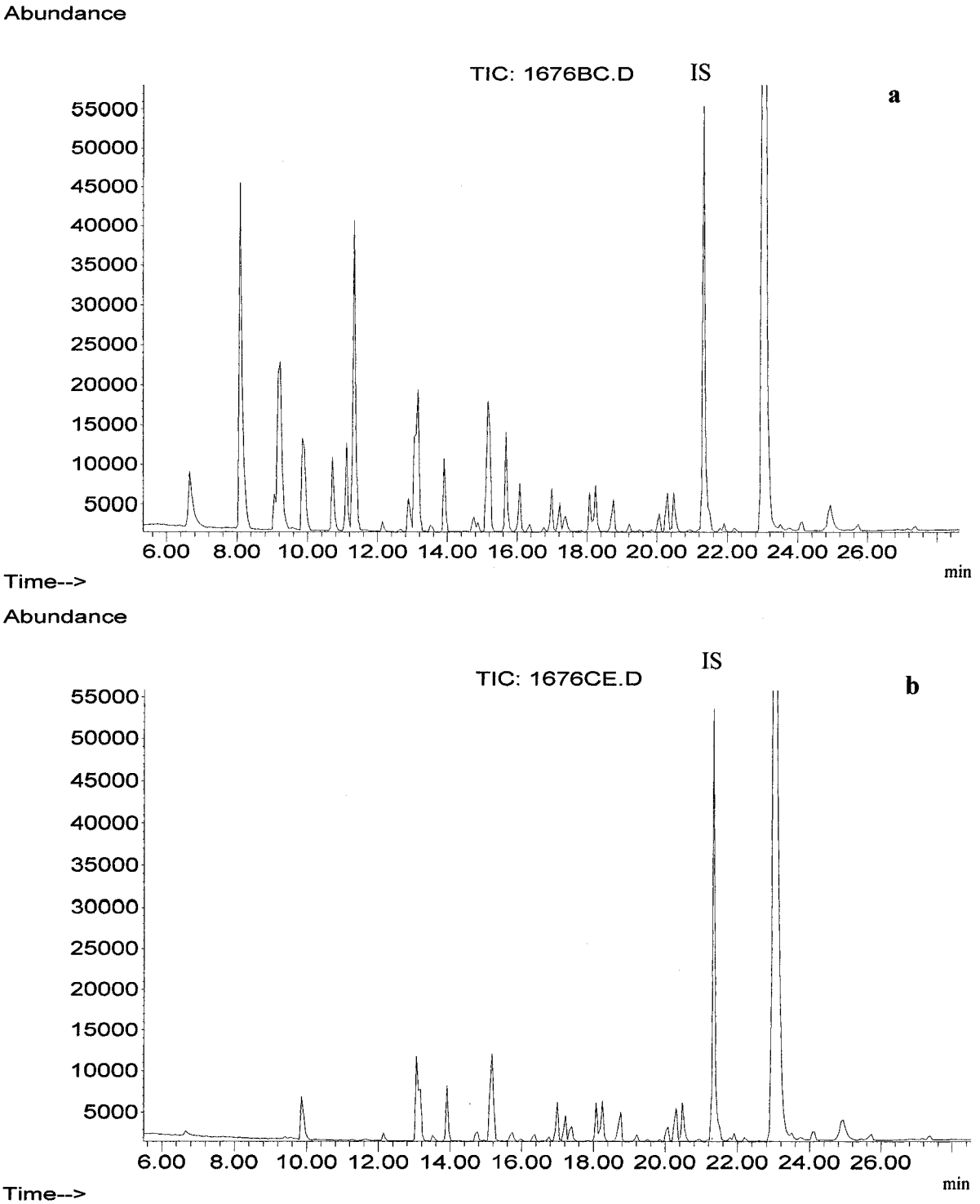


Fig. 3.5. Change in profile of TIC of extracts of cell suspension incubated with Aroclor 1232 at 30 °C for 48 h. a, acid-inactivated cell suspension; b, live cell suspension. Extracts were analyzed with an Agilent GC-MS system.

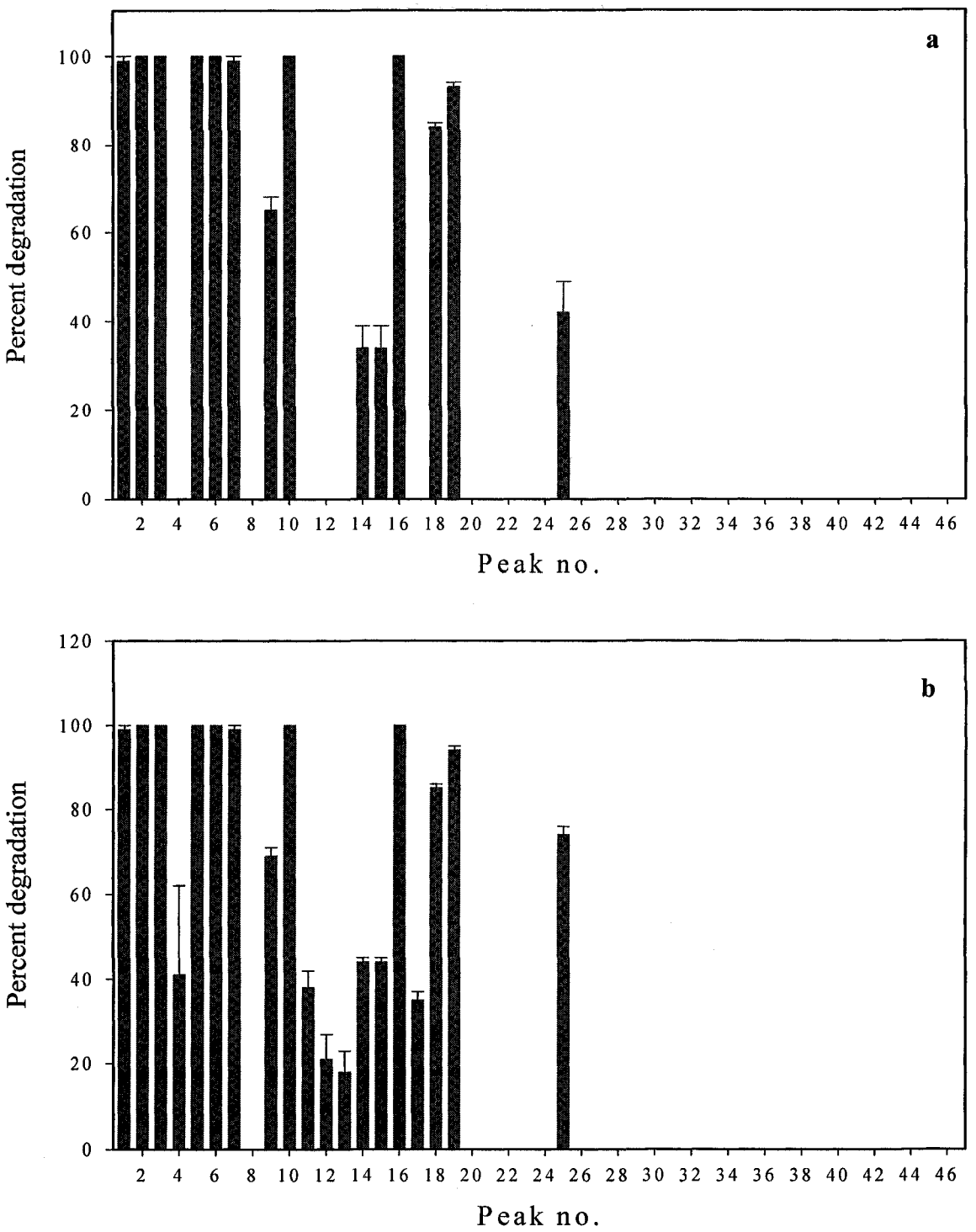


Fig. 3.6. Cometabolic degradation of PCB congeners in Aroclor 1232 by *Hydrogenophaga* sp. IA3-A incubated at 5 ° (a) or 30 °C (b) for 48 h. N = 3; error bars indicates standard deviation. Columns without error bars were totally removed in all replicates.

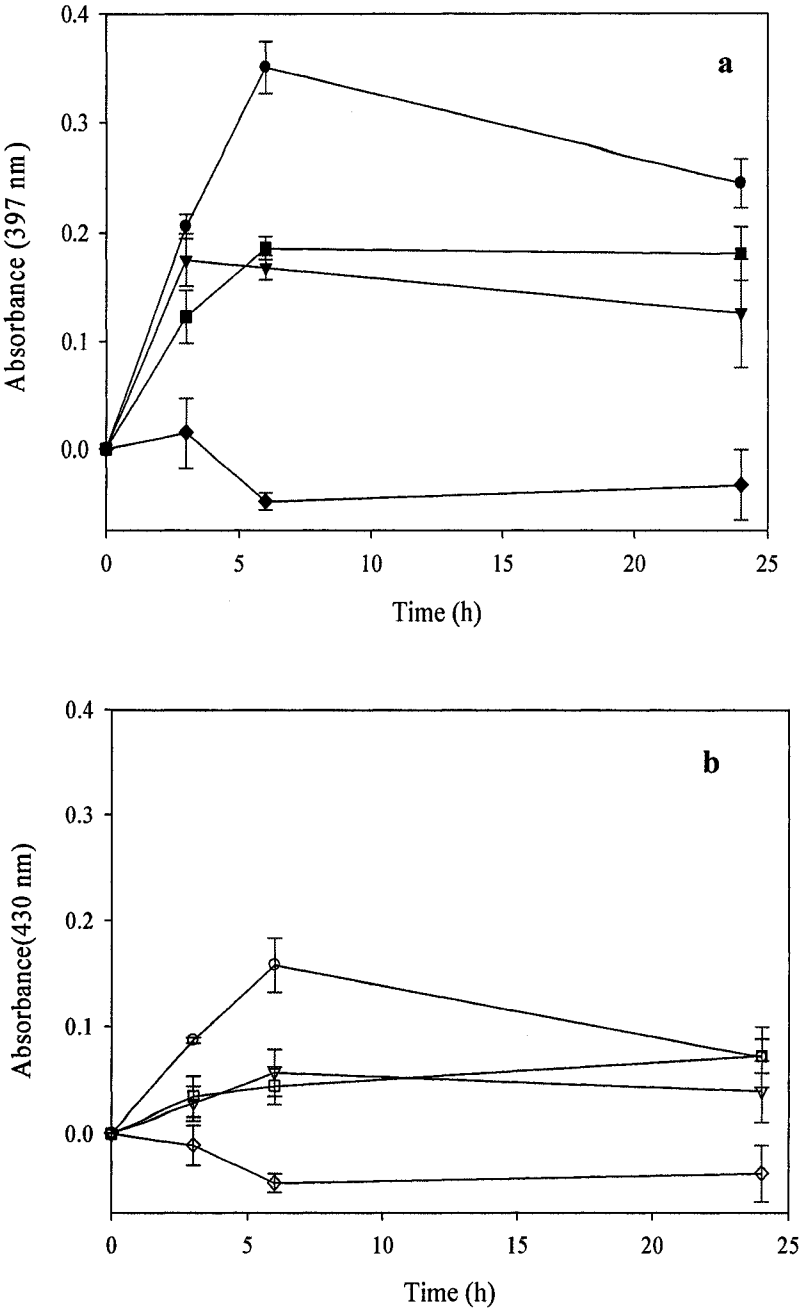


Fig. 3.7. Time-dependent formation of (a) *ortho*- or (b) *para*-chlorinated HOPDAs from PCBs in cell suspension incubated with Aroclor 1221, 1232, 1248, and 1254 at 30 °C. Open and closed circle, Aroclor 1221; open and closed triangle, Aroclor 1232; open and closed square, Aroclor 1248; and open and closed diamond, Aroclor 1254.

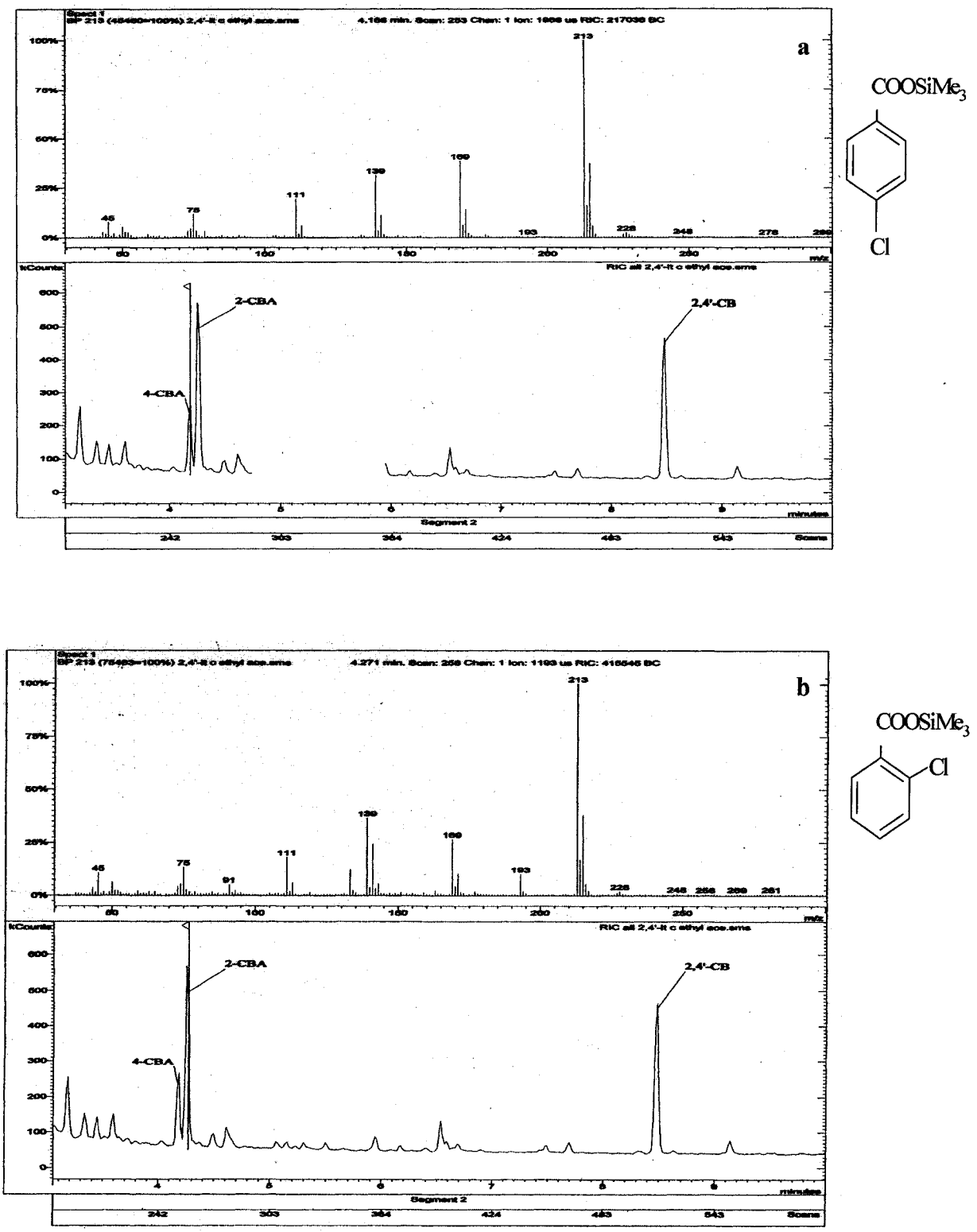


Fig. 3.8. Detection of trimethylsilated (a) 4- and (b) 2-CBA in ethyl acetate extracts of supernatants of cultures incubated with 2,4'-diCB at 5 °C. Note, 2,4'-CB indicates 2,4'-diCB.

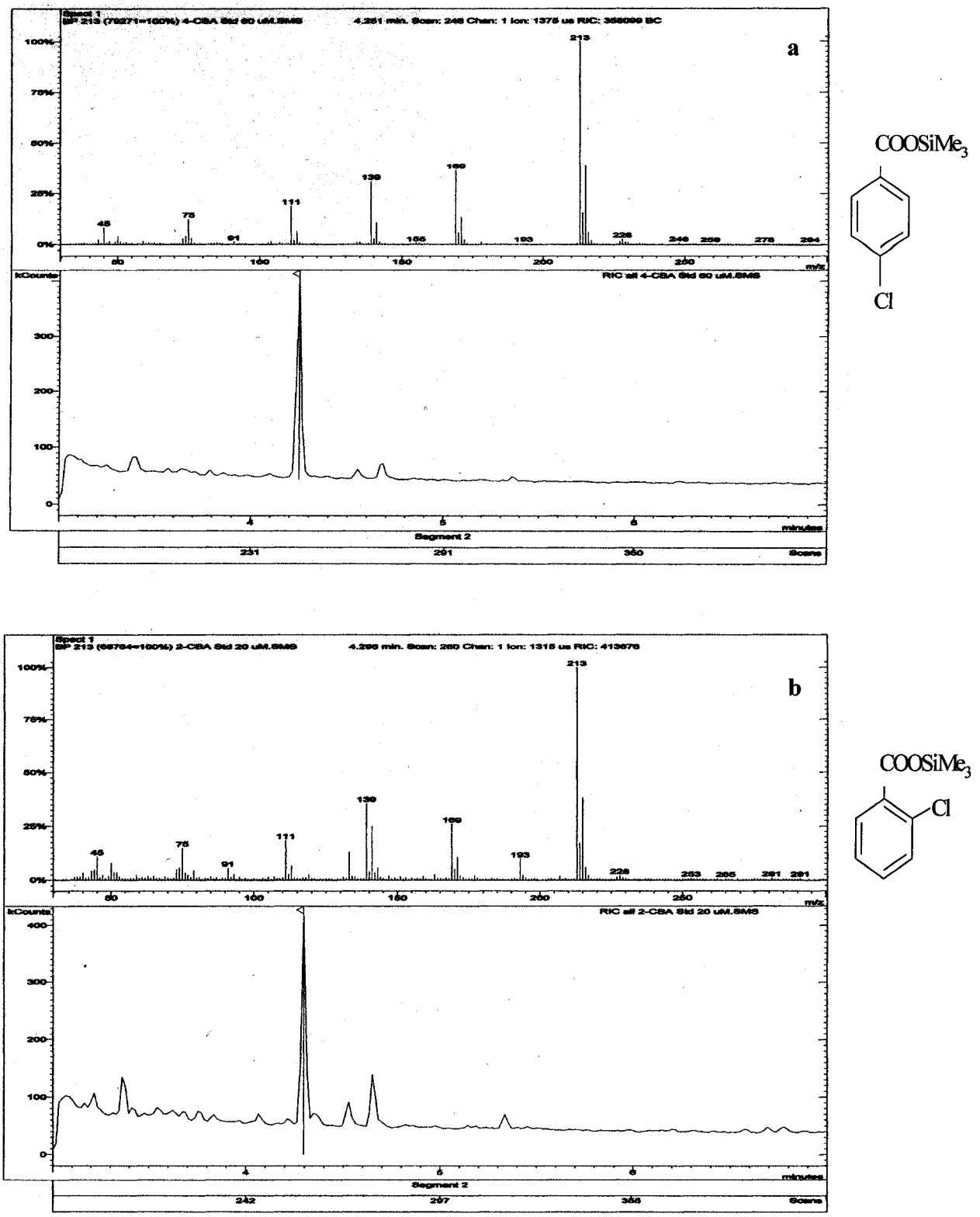


Fig. 3.9. Gas chromatogram and mass spectra of trimethylsilated authentic standards of (**a**) 4-CBA and (**b**) 2-CBA.

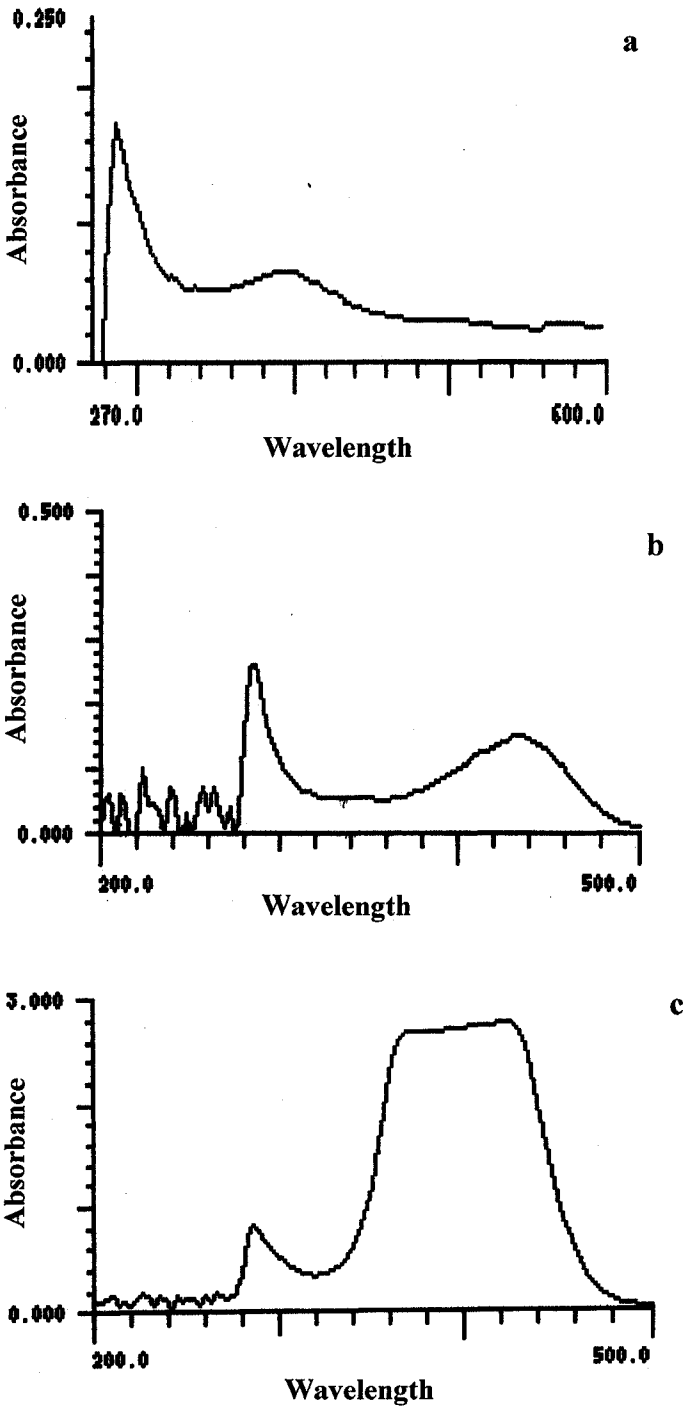


Fig. 3.10. Accumulation of *ortho*- or *para*-substituted HOPDA in supernatants of cell suspension incubated with 2,2'- (a) for 24 h; 4,4'- (b) for 72 h; or 2,4'-diCB (c) for 24 h at 30 °C. UV spectra shown were those that exhibited maximum absorbance over an incubation period of 72 h.

3.4 Discussion

Studies have shown that two processes occur simultaneously during microbial degradation of PCBs in liquid media, the removal of PCBs by microorganisms and possible evaporation of PCBs. As such, the physical loss of PCBs due to evaporation causes frequently false positive results in long-term biodegradation experiments (Vrana *et al.*, 1996). For these reasons, the removal of chlorobiphenyl congeners and PCBs congeners in commercial mixtures by *Hydrogenophaga* sp. IA3-A was determined in aqueous solution in short-term experiments with incubation period of 48 or 72 h. The removal of PCBs in commercial mixtures in aqueous solution at low or moderate temperature by cold-adapted bacteria has been reported before (Master and Mohn, 1998; Mohn *et al.*, 1997), but unlike here, the duration of incubation in those studies was 2 or 5 weeks with strains that are closely related to the genus *Pseudomonas*.

At 5 °C, the extents of removal of monoCBs in Aroclor 1221 were between 63 to 89 %, diCBs were between 30 to 78 %, and the extents of removal of triCBs were between 30 to 75 % (Fig. 3.3). At 30 °C, the extent of removal of each monoCBs was 100 %, diCBs were between 30 to 100 %, and the extents of removal of triCBs were between 27 to 59 % (Fig. 3.3). Except for 2,2'-, 2,6-, and 3,3'-diCBs, all the diCBs were degraded at 30 °C. Though the extent of degradation of each diCB at 5 °C was slightly lower but more diCBs were degraded at 5 °C, including 3,3'-diCB. Eight triCB congeners were degraded at 30 °C, but only six of the congeners were degraded at 5 °C. Results suggest that low temperature probably slow down the degradation of PCBs in Aroclor 1221. Mohn *et al.* (1997) made similar observation using cold-adapted PCB-degrading Arctic soil isolates. Congeners having two *ortho*- chlorine, like 2,2'-, 2,6-diCB, and 2,2',3-, 2,2',5-, and 2,2',6-triCB were resistant

to degradation at 5 ° or 30 °C, but congeners like 2,2'4- and 2,4',6-triCB having two *ortho*-chlorine and a *para*- chlorine were only degraded at 30 °C, suggesting that temperature determines the degradability of these congeners, especially with the knowledge that all the congeners with a *para*- chlorine were degraded at 30 °C. It is known that apart from the number of chlorine, the position of chlorine substituents determines the biodegradability of PCB congeners (Bedard and Haberl, 1990). The observation that congeners like 3,3'-diCB, 2,3,6-, 2,3',6-, and 2,3',5-triCB were degraded at 5 °C and not at 30 °C supports earlier statement that temperature influenced biodegradability of certain congeners. Mohn et al. (1997) reported that 2,2',6-triCB in Aroclor 1221 was degraded at 5 °C in soil slurry experiments but the same congener was not degraded in slurries incubated at 30 °C.

Analysis of the pattern of chlorination of PCBs in Aroclor 1232 that were resistant to degradation and those that were degraded at 5 ° or 30 °C revealed that the likelihood that certain congeners would be removed at low temperature depend, to a large extent, on the pattern of chlorination of the congeners. For example congeners with only double *ortho* (2,2'- and 2,6-diCB) or double *para* substitution (4,4'-diCB) were not degraded at 5 °, but they were moderately degraded at 30 °C (Fig. 3.6). However, substitution of double *para*-chlorinated congener with a single *ortho* chlorine (e.g. 2,4,4'-triCB) or substitution of a congener having two *ortho* chlorine (e.g. 2,6-diCB) on a single ring with a *para*- chlorine on the adjacent ring (e.g. 2,6,4'-triCB) enhanced the removal of the congener at low temperature. Substitution of a congener having two *ortho* chlorine on a single ring with a *meta* chlorine at the 3'- position of the same ring or adjacent ring (e.g. 2,3,6- and 2,6,3'-triCB) did not increase the chances of degradation of the congener at low temperature. This observation is also true if a congener having two *ortho* chlorine (e.g. 2,2'-diCB) on adjacent

rings is substituted with one or more *para* chlorine (e.g. 2,4,2'-triCB), or a 5' *meta* chlorine (2,5,2'-triCB). In addition, substitution of congeners having a 2' *ortho* and 3' or 5' *meta* chlorine on the same or adjacent rings (e.g. 2,3- and 2,3'-, or 2,5-diCB) with a 3'*meta* chlorine (2,3,3'-or 2,5,3'-triCB), rather than a *para* chlorine on the ring adjacent to the one with *ortho*-substitution (e.g. 3,4,2'- and 2,3,4', or 2,5,4'-triCB), reduced the chances of degradation of the congeners at both temperatures, but its effect was more pronounced at 5 °C than at 30 °C. The degradation of 2,4,5,2'-tetraCB at 5 ° or 30 °C was not expected since 2,5,2'-triCB was only moderately degraded at 30 °C. It is likely that substitution of the ring bearing the 2' and 5' chlorines with *para*- chlorine enhanced the removal of the congener at both temperatures. Except in few cases, most of the tetraCBs that were not degraded were characterized by the presence of two *para*- or three *ortho*- chlorines, or the presence of two chlorines on adjacent rings (Table 3.2). Master and Mohn (1998) reported that many of the triCBs and tetraCBs in Aroclor 1242 were generally resistant to degradation after 5 weeks of incubation at 7 °C with the cells of Arctic soil isolate, strain Cam-1. The resistance of most of the triCBs in Aroclor 1221 to degradation at 7 °C after 2 or 5 weeks of incubation was also reported for strain Cam-1 and other Arctic soil isolates (Master and Mohn, 1998; Mohn *et al.* 1997). The extents of degradation of several triCBs in Aroclor 1221 at 5 °C after 48 h by strain IA3-A was between 30 to 75 % (Fig. 3.3).

After 48 h, only 5 and 14 congeners of the 32 PCB congeners that were resolved by GC-MS were removed by cells incubated with Arolcor 1248 at 5 ° and 30 °C, respectively (Table 3.3). PCBs that were degraded at 30 °C included 2,4-, 2,5-, 2,3'-diCB, 2,2',5-, 2,2',4-, 2,2',3-, 2,4',6-, 2,3',4-, 2,4',5-, 2,4,4'-, 2',3,4-TriCB, 2,2',3,4-, 2,3,4',6- and 2,3',4',6-tetraCB. At 5 °C, only 2,3'-diCB, 2,2',4-, 2,2',3-, 2,4',6-, and 2,4,4'-triCB were degraded. At low

temperature, there was insignificant or no removal of congeners with a ring substituted at 2' *ortho* and 5' *meta* position, while the removal of congeners with these pattern of chlorination was moderate at 30 °C. Similar observation was made for congeners with 2' *ortho*, 3 *meta*, plus 4' *para* substitution at 5 °C. The congener that was significantly degraded among the tetraCBs at 30 °C was more likely to be 2,3,4',6-tetraCB, since the congener has a free 2,3 positions on the *para*-substituted ring that was available for dioxygenation. A comparison of the pattern of chlorination of congeners that were degraded at 5 ° and 30 °C indicated that except for those with 2' *ortho* plus 5' *meta* or 2' *ortho*, 3 *meta*, plus 4' *para* substitution that were slightly or moderately degraded at 30 °C, the pattern was generally similar at both temperatures.

As observed here, resistance of 2,2'- and 2,6-diCB, and in few instances, 4,4'-diCB, to degradation at low temperature has been reported before (Master and Mohn, 1998). It is interesting to note that some congeners in Aroclor 1232 that were resistant to degradation at 5 °C (e.g. 4,4'-diCB, 2,3,6- and 2,6,3'-triCB) were moderately degraded in Aroclor 1221 by strain IA3-A, while congeners like 2,3,4'- and 3,4,2'-triCB that were significantly degraded in Aroclor 1232 were moderately degraded or not degraded in Aroclor 1221. This difference could be due to different composition of PCB congeners in Aroclor 1221 and Aroclor 1232. Similar observation was reported for the removal of 2,2'- and 2,6-diCB in Aroclor 1221 and Aroclor 1242 at 7 °C by bacterial strain Cam-1 (Master and Mohn, 1998). It is known that degradation of any one congener in a mixture could be influenced by the other congeners present in the mixture (Bedard *et al.*, 1986).

Results from experiments using the pure congeners confirmed the results obtained with the PCBs mixtures. As shown for the PCB mixtures that 2,2'- and 2,6-diCB were resistant to

degradation in most cases, and that 4,4'-diCB was only moderately degraded in some cases, separate experiments with pure 2,2'- and 2,6-diCB showed that strain IA3-A did not degrade the congeners to significant extents at 5 ^{o} or 30 ^{o}C. Likewise, moderate removal of 4,4'-diCB was only observed at 30 ^{o}C, while the congener was not degraded at 5 ^{o}C. Though, it is known that most bacterial strains with limited ability to degrade 2,2'-diCB could degrade 4,4'-diCB efficiently (Bedard *et al.*, 1986). It appear that strain IA3-A does not belong to this group of PCB-degrading bacteria. However, as shown before (Bedard *et al.*, 1986), results here suggests that chlorination of *para-* substituted congeners at *ortho* positions enhanced the removal of these congeners, considering the observation that a congener like 2,4,4'-triCB was significantly degraded in most cases while 4,4'-diCB, a congener with less chlorine was only moderately degraded in several cases.

Monochlorobiphenyls like 2- and 4-monoCBs were shown to induce biphenyl dioxygenase gene in cells of psychrotolerant *Pseudomonas* strain Cam-1 (Master and Mohn, 2001). Results here also show that biphenyl dioxygenase activity was higher in the extracts of cells grown on succinate and 2,4'-diCB than in the extracts of cells grown on succinate alone (Table 3.5). This is an indication that a dichlorobiphenyl compound could induce biphenyl dioxygenase activity in the cells of strain IA3-A. Inability of strain IA3-A to significantly degrade 2,2'-diCB and 2,5,2'-triCB in Aroclor 1221 and Aroclor 1232, and its failure to degrade 2,3,2',5'-tetraCB (Table 3.2, peak no. 27) in Aroclor 1232 and 1248 suggest that the bacterium does not possess a biphenyl dioxygenase enzyme that is of capable of attacking PCBs at carbon positions 3 and 4 (Bedard *et al.*, 1987a). Most PCB-degrading bacteria are known to utilize biphenyl dioxygenase that is only capable of attacking chlorinated biphenyls at carbon positions 2 and 3 (Bedard *et al.*, 1986).

Measurements of formation of ring fission products at 397 nm and 430 nm (Seeger *et al.* 1995) showed that except for Aroclor 1254, *ortho*-chlorinated HOPDA(s) were produced in abundance from the Aroclor mixtures (Fig. 3.7). Formation of *para*-chlorinated HOPDA(s) was low when compared to the formation of *ortho*-chlorinated HOPDA(s). This may indicate that oxidation of PCB congeners in the mixtures favoured the formation of *ortho*-chlorinated HOPDA(s) than the formation of *para*-chlorinated HOPDA(s). Formation of *ortho*- or *para*-substituted HOPDA from chlorinated biphenyls indicates the regiospecificity of oxidation of PCBs (Seegar *et al.*, 1995). In addition, excess accumulation of HOPDA with absorption maximum at 397 nm from 2,4'-diCB (Fig. 3.11) suggest that strain IA3-A preferentially oxidized the *para*-substituted ring of the congener, in agreement with previous reports (Maltseva *et al.*, 1999; Bedard and Haberl, 1990). These data indicates that *Hydrogenophaga* sp. IA3-A likely oxidized some of the congeners in the PCB mixtures at the ring bearing *para* chlorine rather than the ring with *ortho* chlorine.

Lack of growth on CBAs and failure of biphenyl-grown cells to oxidize CBAs suggests that biphenyl grown cells does not contain the enzyme system that is required for the oxidation of CBAs (Table 3.4) or that transport of CBA into cells require a factor that was absent in biphenyl-grown cells since limited oxidation of some CBAs was observed with benzoate grown cells. Thus, CBAs could be regarded as dead-end products of transformation of PCBs by strain IA3-A, in agreement with previous reports on most PCB-degrading bacterial strains (Kim and Picardal, 2000; Ahmed and Focht, 1973).

Chapter 4

Temperature-Dependent Biotransformation of 2,4'-Dichlorobiphenyl by *Hydrogenophaga* sp. IA3: Higher Temperatures Prevent Excess Accumulation of *Meta*-Cleavage Products

4.1 Introduction

A majority of PCB-contaminated sites that require treatment are contaminated with highly chlorinated PCB mixtures that are resistant to biodegradation. Except for *Burkholderia xenovorans* LB400 (formerly *Burkholderia cepacia* LB400) (Mondello, 1989), *Alcaligenes eutrophus* strain H850 (Bedard *et al.*, 1987a, 1987b), and *Rhodococcus* sp. strain RHA1 (Seto *et al.*, 1995) that degrades highly chlorinated congeners, most PCB-degrading aerobic bacteria can only remove the lightly chlorinated congeners. Thus, the most promising approach for biological treatment of these sites is a sequential anaerobic-aerobic scheme, where highly chlorinated congeners are anaerobically dechlorinated to produce lightly chlorinated congeners that can be degraded aerobically (Quensen *et al.*, 1988).

The major by-products of anaerobic dechlorination of PCBs are *ortho*-, and *ortho*- plus *para*-substituted congeners that include 2-, 4-monoCB, 2,4-, 2,6-, 2,2'-, 2,4'-diCB, 2,2',4-, and 2,4,4'-triCB (Quensen and Tiedje, 1998; Quensen *et al.*, 1988). Therefore, the aerobic phase of the anaerobic-aerobic scheme would require bacteria that are capable of degrading *ortho*- and/or *ortho*- plus *para*-substituted congeners. Studies have reported the aerobic degradation of the by-products of anaerobic dechlorination of PCBs (Maltseva *et al.*, 1999; Harkness *et al.*, 1993), although most PCB-degrading strains could only transform lightly chlorinated *ortho*- plus *para*-substituted congeners to products that include *meta*-cleavage

compounds (i.e. 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoic acids, HOPDAs) as the major intermediates (Billingsley *et al.*, 1999; Bruhlmann and Chen, 1999; Maltseva *et al.*, 1999; Bedard and Haberl, 1990; Furukawa *et al.*, 1979a, 197b).

Maltseva *et al.* (1999) proposed that *ortho* plus *para*-substituted congeners like 2,4'-diCB and 2,4,4'-triCB can be used to identify bacteria having the ability to transform PCBs more efficiently. This class of congeners can be used to discriminate the specificity of attack of PCBs by screening for the accumulation of chlorobenzoates and HOPDAs. Some strains extensively degraded 2,4'-diCB into 4-CBA, while other strains transformed 2,4'-diCB mostly into HOPDA. *Rhodococcus erythreus* NY05 efficiently attacked both rings of 2,4'-diCB, but only 10 % of the degraded compound was recovered as CBAs (Maltseva et al., 1999). HOPDA was the major product of oxidation of 2,4'-diCB by *Alcaligenes* sp. Y42 and *Rhodococcus sp.* P6 (formerly *Acinetobacter* sp. P6) (Furukawa *et al.*, 1979a). Some bacterial strains attacked the 4-chlorophenyl ring of 2,4'-diCB, converting less than 15 % of the degraded compound to 2-CBA, while two strains converted 90 to 100 % of the congener to 4-CBA (Bedard and Haberl, 1990).

In chapter three (section 3.3.4.), it has been shown that *Hydrogenophaga* sp. IA3-A also accumulates excess level of HOPDA from 2,4'-diCB. The objective of the present chapter is to investigate if temperature determines the pattern of transformation of 2,4'-diCB. It is assumed that it would be possible to prevent excess accumulation of HOPDA from 2,4'-diCB if cells of strain IA3-A that contain multiple pathways for transformation of PCBs are pre-treated or incubated at different temperatures. The metabolic products that are formed from 2,4'-diCB at different temperatures would be compared and relative production of key intermediate compounds and end-products will be determined. In the absence of appropriate

standards of the possible metabolic products, the types and numbers of intermediates that are formed from 2,4'-diCB by *Hydrogenophaga* sp. IA3-A were compared to results of previous investigations (Maltseva *et al.*, 1999; Ahmad *et al.*, 1991; Furukawa *et al.*, 1979a) of metabolism of the compound.

4.2 Materials and Methods

4.2.1 ***Chemicals.*** 2,4'-diCB (99.4 % purity) and 2,4,4'-triCB (100 % purity) were purchased from Crescent Chem. Co. (New York, NY, USA). 2- (95+ %), 4-CBA (95+ %), and 2,2',4,4',6,6'-hexaCB (97+ %) were obtained from Ultra Scientific (RI, USA). *N*-O-bis-(trimethylsilyl)-trifluoroacetamide containing 1% trimethylchlorosilane (TMS) and other solvents were purchased from Sigma-Aldrich Chem. Co. (St. Louis, MO, USA). All other chemicals were purchased from other commercial distributors and were of analytical grade.

4.2.2 ***Source of organism and culture conditions.*** The organism was isolated from PCB-contaminated soil samples and it was characterized and subsequently identified as a *Hydrogenophaga* sp. IA3-A (Chapter 2) (Lambo and Patel, 2006a; 2006b). The optimal pH and substrate concentration for oxidation of 2,4'-diCB by cells of *Hydrogenophaga* sp. IA3-A in 0.05 M sodium phosphate buffer were described in Chapter 2 (section 2.3.2). Biphenyl-degrading enzymes were induced in cells of *Hydrogenophaga* sp. IA3-A by growing cells in MSM containing 0.5 % (w/v) of biphenyl following the procedures described in Chapter 2 (section 2.2.2).

4.2.3 ***Transformation of model ortho- plus para-substituted congeners.*** To determine the possible transformation products of 2,4,4'-triCB, 2 ml of biphenyl-grown cell suspensions with a final $OD_{600\ nm}$ of 1.0 were transferred into acid-washed 25 ml tubes. Thereafter, 2,4,4'-

triCB was added into the suspension as a acetone solution to a final concentration of 100 μM. The reactions were incubated at 5 or 30 °C on a shaker at 200 rpm for 72 h.

To determine the transformation products of 2,4'-diCB at different temperatures, 1 ml of biphenyl-grown cell suspension was dispensed into 4 ml glass vials. Cells in control set-up were inactivated with one drop of 70 % perchloric acid. Then, 2,4'-diCB was added into the control and experimental vials to a final concentration of 250 μM. The vials were closed with Teflon-lined screw caps and incubated immediately at 10 °, 25 °, 37 ° or 45 °C on a shaker at 200 rpm for 48 h. In addition, time points were taken at 4, 12, or 24 h to detect transient intermediates. Reactions were frozen at the end of the incubation period and stored at -20 °C.

4.2.4 ***Sensitivity of transformation of 2,4'-diCB to temperature.*** To investigate the sensitivity of transformation of 2,4'-diCB to different temperature treatments, the procedures outlined below were followed. Cells that were grown on 0.5 % (w/v) of biphenyl at 30 °C were washed in 0.05 M phosphate buffer, (pH 7.5) and the cells were suspended in 5 ml of the same buffer. The cell suspension was transferred into 1 L flasks containing 500 ml of MSM and 0.5 % (w/v) of biphenyl and the cultures were incubated further at 10 ° or 37 °C for 48 h. Thereafter, cells were harvested by centrifugation, washed and suspended in 0.05 M sodium phosphate buffer (pH 7.5) as described in Chapter 2 (section 2.2.2). Two milliliter of the cell suspension was transferred into acid-washed sterile tubes, and acetone-solution of 2,4'-diCB was added into the suspension to a final concentration of 250 μM. Cells in control set-up were inactivated with one drop of 70 % perchloric acid prior to the addition of 2,4'-

diCB. The cell suspensions were incubated at 10 °, 25 °, and 37 °C on a shaker at 200 rpm for 48 h, and reactions were stopped as described above.

4.2.5 ***Effect of temperature on HOPDA.*** Six milliliters of biphenyl-grown cell suspension was placed in a 25 ml tube, and the suspension was supplemented with 250 μM of 2,4'-DiCB. The culture was incubated at 25 °C for 12 h to allow the accumulation of HOPDAs. Thereafter, the culture was centrifuged and the supernatant was collected. Then, freshly prepared biphenyl-grown cells were suspended in 1.2 ml of the supernatant at an $OD_{600\ nm}$ of 2.0, followed by incubation at 10 °, 25 °, 37 °, or 45 °C for 48 h at 200 rpm. Cell-free supernatant that was prepared as above was also incubated separately at 10 ° or 45 °C for 24 h. The stability (i.e. loss) of HOPDAs in culture supernatants or cell-free supernatants was monitored by GC-MS and UV spectrometry.

4.2.6 ***Analytical methods.*** The presence of inorganic chloride (from dechlorinaton of 2,4'-diCB) in supernatants was confirmed by the $AgNO_3$ precipitation method (Adriaens *et al.*, 1989). One millilitre of sample acidified with 5 µL of concentrated HNO_3 was centrifuged to separate cells. Then, optical density was read at 330 nm after the addition of 10 µL of 0.1 M $AgNO_3$ into the supernatant. Accumulation of HOPDAs was monitored by visible spectral scanning of supernatants by following the method of Seeger *et al.* (1995) using a Shimadzu UV-260 spectrophotometer or Spectronic Genesys-5 spectrophotometer (Milton Roy Co., USA). Absorbance was measured at the λ_{max} (397 nm) that was determined by visible

spectral scanning of supernatants of cultures that was incubated with 2,4'-diCB (Lambo and Patel, 2006a).

The internal standard, 2,2',4,4',6,6'-hexaCB (17.31 μM) was added into the samples to be analyzed for the removal of 2,4'-diCB. Thereafter, 100 µL of 10 % Triton X-100 solution was added into each sample followed by the addition of 2 ml of hexane and acetone (1/1, v/v) and 0.5 g of $NaSO_4$. The samples were extracted at 250 rpm for 24 h and extracts were transferred into glass vials. One microlitre of each extract was injected by an auto-injector using the Agilent GC-MS system and the conditions described in Chapter 2 (section 3.2.7), except that the temperature program ran from 90 °C to 100 °C at 5 °C min^{-1}, and then to 140 °C at 5 °C min^{-1} for 1 min, and to 230 °C at 10 °C min^{-1} and held for 10 min. The 2,4'-diCB in the experimental and control samples was quantified using a calibration curve that was constructed with standards bracketing the concentration expected in samples showing no degradation.

CBAs were analyzed by HPLC according to the method of Schwein *et al.* (1988) using an Agilent series 1000 chromatograph equipped with a multiple wavelength detector set at 230 nm. Separation was done on a reversed-phase Bondalone C-18 column (internal diameter 3.9 mm and length 150 mm; Phenomenex, CA., USA) with a solvent system made up of 20 mM phosphoric acid containing 30 % (v/v) acetonitrile.

Metabolic products in the samples were also analyzed by GC-MS. Two volumes of hexane were added into each sample at neutral pH and non-polar metabolites were extracted at 250 rpm for 24 h. The hexane extracts were collected and the aqueous phase were acidified to pH 3 with 1.0 M HCl. Polar metabolites were extracted with two volumes of ethyl acetate at 250 rpm for 24 h. The hexane and ethyl acetate extracts were combined and dried over

Na_2SO_4. The extracts were evaporated and the residues were derivatized by a modification of the method of Maltseva *et al.* (1999) using 20 µL of pyridine and 80 µL of *N,O*-bis(trimethylsilyl)trifluoroacetamide containing 1 % trimethylchlorosilane (TMS). From each sample, 1 µL aliquot was injected by an auto-injector and analyzed using the Agilent GC-MS system that is described in Chapter 2 (section 3.2.7). The carrier gas was helium at a flow rate of 1 ml min^{-1} with a head pressure of 10.75 psi. The system was operated in full scan mode from 50 to 500 amu. The temperature of the injector and the transfer line was 260 and 280 °C, respectively. The electron impact (EI) mass spectra were measured at 70 eV (ionization potential). The column temperature was increased from 90 °C to 170 °C at 5 °C min^{-1}, and then increased at 15 °C min^{-1} to 320 °C and held for 10 min. The GC-MS profile of the metabolic products formed from 2,4-diCB or 2,4,4'-triCB by strain IA3-A were compared to the products described in previous reports (Flanagan and May, 1993; Ahmad *et al.*, 1989; Masse *et al.*, 1989; Furukawa, 1979a; 1979b).

4.3 Results

4.3.1 ***Biotransformation of 2,4'-CB at different temperatures.*** *Hydrogenophaga* sp. IA3-A transformed 2,4'-diCB to 2- and 4-CBA, HOPDAs, and inorganic chloride as shown in Table 4.1. Also detected in culture extracts were several chlorinated intermediates (Table 4.2, Fig. 4.1 – 4.4). After 48 h, the extents of degradation of 250 μM of 2,4'-diCB in the cultures were between 46 to 195 μM. The extents of degradation of 2,4'-diCB were higher, and not significantly different in cultures incubated at 10 °, 25 °, and 37 °C. In contrast, extent of degradation of the congener was much lower in cultures incubated at 45 °C. Cells in the acid-inactivated control cultures failed to degrade 2,4'-diCB. The recovery of 2,4'-diCB at 10 °, 25 °, 37 °, and 45 °C was 80, 77, 90, and 91 %, respectively.

4.3.2 ***Production of 2- and 4-CBA at different temperatures.*** Strain IA3-A attacked 2,4'-diCB at various temperatures to produce 2- and 4-CBA in different amounts (Table 4.1). Recovery of degraded 2,4'-diCB as CBAs at low and moderate temperatures, was between 23 to 27 %. In contrast, 87 % of the degraded compound was recovered as CBAs at 45 °C. The ratio of 4- to 2-CBA at 10 °, 25 °, and 37 °C was 10.5, 7.7, and 4.4, respectively. The ratio of 4- to 2-CBA in cultures incubated at 45 °C was 8.7. The recovery of degraded 2,4'-CB as 4-CBA was higher in cultures incubated at 45 °C when compared to the cultures incubated at lower temperatures (Table 4.1). CBAs are known to be produced from HOPDAs (Seeger *et al.*, 1995; Furukawa *et al.*, 1979b). The production of 2- and 4-CBA here suggests that the two compounds were end-products of transformation of 2,4'-diCB. Previous

experiments showed that 2- and 4-CBA are not oxidizable substrates for biphenyl-grown cells of strain IA3-A (Chapter 3, See Table 3.4; Lambo and Patel, 2006b). In addition, the rates of oxidation of 2- and 4-CBA by benzoate-grown cells were very low. It is unlikely that 2- and 4-CBA were converted further once they are formed.

4.3.3 ***Accumulation and stability of HOPDAs.*** Time-dependent formation of HOPDA(s) from 250 μM of 2,4'-diCB at 25 °C showed that HOPDA(s) were detected as early as 5 min (Fig. 4.6). Relatively higher level of the compound(s) was present in culture broths after 30 min. The level of the compound(s) in culture broths peaked after 4 h, and there was no appreciable decrease of absorbance from 4 – 72 h. A comparison of the UV spectra showed that the spectra of supernatants of cultures incubated for 4, 12, and 72 h were generally broad (probably due to very high concentration of HOPDAs), while the spectra of supernatants of cultures incubated for shorter periods were less broad. Likewise, the spectrum of supernatant of a culture incubated at 45 °C for 5 min with subsequent incubation at 25 °C for 48 h was narrower (Fig. 4.7). In spite of these differences, the wavelength of maximum absorption was always 397 nm.

Several isomeric *ortho*-chlorinated HOPDAs were detected by GC-MS in the extracts of cultures incubated at low and moderate temperatures (Table 4.2, Fig. 4.1 - 4.3), but only one HOPDA compound was detected in the extracts of cultures incubated at 45 °C (Table 4.2 and Fig. 4.4). Mass spectral data indicates that all the HOPDAs were structurally similar to the *ortho*-substituted HOPDA that is formed from 2,4'-diCB by some mesophilic strains (Maltseva *et al.*, 1999; Furukawa *et al.*, 1979a). The detection of isomeric HOPDAs in most

of the extracts was unexpected since Maltseva *et al.* (1999) and Furukawa *et al.* (1979a) reported that only one HOPDA compound accumulated from 2,4'-diCB. The fact that the derivatization procedures employed here are similar to the procedures employed by Maltseva *et al.* (1999) ruled out the possibility that the isomeric HOPDAs were formed almost exclusively by intramolecular rearrangement during derivatization. Mass spectral data show that all the HOPDAs contained two chlorine atoms. This confirms that the compounds contained *ortho-* chlorine and that measurement of absorbance of supernatant at 397 nm should indicate the level of accumulation of the compounds as proposed by Seeger *et al.* (1995). HOPDAs accumulated to higher levels in the cultures incubated at 10 °, 25 °, or 37 °C when compared to the cultures incubated at 45 °C (Table 4.1). The extents of accumulation of HOPDAs were generally similar at low and moderate temperatures. The extent of accumulation of HOPDA at 45 °C was more than sixty-fold lower when compared to the extent of accumulation of the compounds at 10 °, 25 °, or 37 °C. In separate experiments, the absorbance of supernatant of cultures that degraded 45 μM of 2,4'-CB at 25 °C was 1.08 after 24 h (see UV spectra in Fig. 4.7). This confirmed that absence or low level of HOPDA(s) (Fig. 4.7) in cultures incubated with 250 μM of 2,4'-diCB at 45 °C (Table 4.1 and Table 4.2) was not due to low extent of transformation of 2,4'-diCB at this temperature.

HOPDAs were resistant to further degradation at all temperatures investigated. The compounds were stable in cell-free supernatants and they were not degraded further in supernatants incubated with freshly prepared cells. GC-MS profile of the compounds did not change during incubation period. These shows that HOPDAs that were formed from 2,4'-diCB were stable, in agreement with previous reports (Maltseva *et al.*, 1999; Bedard and

Haberl, 1990). These data also suggests that low level of accumulation of HOPDA in cultures that transformed 46 μM of 2,4'-diCB at 45 °C (Table 4.1) was not due to abiotic reactions.

4.3.4 ***Formation of hydroxylated intermediates.*** Several isomeric hydroxylated intermediates were detected in extracts of cultures incubated with 2,4'-diCB (Table 4.2; Fig. 4.1 - 4.4; see appendix B). As with biphenyl-grown cells, cells grown on acetate and pyruvate produced multiple isomeric products from 2,4'-diCB (Fig. 4.5). Analysis of the mass spectra of the compounds suggests that most of them were structurally similar to previously characterized products of 2,4'-diCB metabolism (Ahmad *et al.*, 1991; Furukawa *et al.*, 1979a). But unlike here these authors did not report the formation of multiple isomeric products from 2,4'-diCB. Also detected were three isomers of monochloro-hydroxybiphenyl (Table 4.2). This is the first study to report the formation of monochloro-hydroxybiphenyl from 2,4'-diCB, though these products are usually produced from monoCBs (Flanagan and May, 1993; Masse *et al.*, 1989). GC-MS analysis of the stock acetone solution of 2,4'-diCB indicates that the solution did not contain any contaminants (Fig. 4.8). The mass spectra of the intermediates detected in the extracts of cultures of biphenyl-grown cells incubated with 2,4'-diCB are presented in the appendix (see appendix C)

As with 2,4'-diCB, several isomeric hydroxylated intermediates were detected in the extracts of cultures incubated with 2,4,4'-triCB (Table 4.3). Also detected were three isomeric compounds with similar mass spectra as the only trichlorinated-HOPDA that is formed from 2,4,4,'-triCB by certain mesophilic strains (Furukawa *et al.*, 1979b; Maltseva *et*

al., 1999). The total ion chromatogram of culture extracts revealed that one of the HOPDAs was the most abundant metabolite in culture extracts.

4.3.5 ***Sensitivity of 2,4'-DiCB transformation to temperature.*** Results of experiments conducted using biphenyl-grown cells pre-treated at 10 ° or 37 °C, prior to incubation with 2,4'-diCB at various temperatures, showed that cells pre-treated at 10 °C retained the ability to extensively degrade 2,4'-diCB at 10 ° and 25 °C (Table 4.4). Extracts of cultures incubated at 10 ° or 25 °C after pre-treatment at low temperature also contained several isomers of HOPDAs. But only one HOPDA compound was detected in the extracts of the cultures incubated at 37 °C (Fig. 4.9). Cells pre-treated at 37 °C failed to degrade 2,4'-diCB at 10 °C (Table 4.4), and these cells were almost inactive against 2,4'-diCB at 25 °C, forming only trace levels of CBAs that could not be integrated by GC. Interestingly, cells pre-treated at 37 °C degraded 2,4'-diCB when incubated at 37 °C, but these cells converted the compound to products that were not extractable using the method employed (Table 4.4 and Fig. 4.10). The observation that 2,3'-CBA that was added into the culture extracts prior to derivatization was present in the chromatogram suggest that the derivatization process was complete, and that absence of any of the expected products in the chromatogram was not due to incomplete derivatization.

Table 4.1 Degradation of 2,4'-diCB and production of metabolites at various temperatures by biphenyl grown cells of *Hydrogenophaga* sp. IA3-A incubated for 48 h.

Temperature (° C)	2,4'-CB[a] degraded (μM)	Chloride ion	Percent of 2,4'-DiCB recovered as CBA		Accumulation[b] of HOPDA(s) ($OD_{397\ nm}$)
			2-CBA	4-CBA	
10	195 ± 12	+	2	21 ± < 1	2.57
25	190 ± 19	+	3 ± 1	23 ± 1	2.55
37	190 ± 10	+	5 ± 1	22 ± 2	2.45
45	46 ± 2	+	9 ± 1	78 ± 5	0.04

Note: $n = 3 \pm$ SD except where stated otherwise

[a]Initial concentration of 2,4'-diCB was 250 μM. Statistical analysis performed using *t*-test; statistical significant difference is defined as $p < 0.05$; extents of degradation at 10 °, 25 °, and 37 °C ($p > 0.05$); degradation at 45 °C compared to 10 ° , 25 °C , or 37 °C ($p < 0.05$); $n = 3$

[b]Absorbance of supernatants of cultures incubated with 250 μM of 2,4'-CB for 48 h using a Shimadzu UV-260 spectrophotometer; $n = 1$.

+, detected (detection limit for chloride, 5.31 μM).

Table 4.2 Products of metabolism of 2,4'-diCB at different temperatures by biphenyl-grown cells of *Hydrogenophaga* sp. IA3-A as detected by GC-MS

	m/z[a]			No. of products detected[b]			
Metabolic products[c]	M^+	M-Me	M-Cl	10 °C	25 °C	37 °C	45 °C
Monochloro-hydroxybiphenyls	276	261	241	3	3	3	3
Dichloro-hydroxybiphenyls	310	295	271	2	2	2	2
Monochloro-dihydroxybiphenyls	364	349	329[d]	5	5	5	4
Dichloro-dihydroxybiphenyls	398	383	-	1	1	1	1
Dichloro-dihydrodiols	400	395	365	3	3	3	2[e]
HOPDAs	430[f]	415	395	7	7	7	1

[a]Based on fragment containing ^{35}Cl only (M^+, molecular ion; M-Me, M^+ minus CH_3; M-Cl, M minus Cl).

[b]Metabolites were detected in extracts of cultures incubated with 250 μmol l^{-1} of 2,4'CB for 4, 12, or 48 h

[c]Peak nos. of metabolites on total ion chromatogram (TIC): monochloro-hydroxybiphenyls, peak nos. 3, 4, and 5; dichloro-hydroxybiphenyls, peak nos. 6 and 8; monochloro-dihydroxybiphenyls, peak nos. 7, 9, 10, 11, and 12; dichloro-dihydroxybiphenyls, peak no 16; dichloro-dihydrodiols, peaks 13, 14, and 15; HOPDAs, peak nos. 17, 18, 19, 20, 21, 22, and 23. (See the TIC in Figure 4.1 - 4.4; and the mass spectra of the proposed compounds in appendix B)

[d]Low abundance to almost non-detectable

[e]One of the products was detected at trace level

[f]The molecular ion at *m/z* 430 was not detected in one of the seven products. Fragmentation pattern of all seven compounds were generally similar (see appendix B).

Table 4.3 GC-MS data of TMS derivatives of metabolites detected in the extracts of cultures of *Hydrogenophaga* sp. IA3-A incubated with 2,4,4'-triCB at 5 °C for 72 h

Peak no.	*m/z*[a]								
	M^+	M-Me	M-Cl	Other ions					
1	228	213	-	139[a]	111[c]	75	73		
2	262	247	227	173[a]	145[c]	75	73		
3	398	383	363	310[a]	281[c]	175	173	75	73
4	398	383	-	310[a]	281[c]	175	173	75	73
5	398	383	363	310[a]	281[c]	175	173	75	73
6	398	383	-	310[a]	281[c]	175	173	75	73
7	398	383	-	310[a]	281[c]	175	173	75	73
8	398	383	-	310[a]	281[c]	175	173	75	73
9	434	419	399	345[a]	317[c]	147	145	75	73
10	434	419	399	345[a]	317[c]	147	145	75	73
11	434	419	399	345[a]	317[c]	147	145	75	73
12a	464	449	429	375[a]	347[c]	175	173	75	73
12b	464	449	429	375[a]	347[c]	175	173	75	73
12c	464	449	429	375[a]	347[c]	175	173	75	73

[a]Calculation of *m/z* based on fragment containing ^{35}Cl only.

[b]Indicates fragments containing ($M-SiMe_3O^+$)

[c]Indicates fragment containing ($SiMe_3O_2C^+$).

Note: Peak 1, 4-CBA; Peak 2, 2,4-CBA; Peaks 3, 4, 5, 6, 7, and 8, dichlorodihydroxybiphenyls; peaks 9, 10, and 11, trichloro-dihydrodiols; Peaks 12a, 12b, and 12c, trichlorinated-HOPDAs. (See complete fragmentation pattern in appendix C).

Table 4.4 Degradation of 2,4'-diCB and metabolites production by cells of *Hydrogeno-phaga* sp. IA3-A pre-treated at different temperature

Pre-treatment and incubation Temperature (°C)	2,4'-diCB[a] degraded (μM)	Metabolites detected			
		2-CBAs	4-CBA	Hydroxylated products	HOPDA(s)[b]
Pre-treated at 10 °C					
10	238 ± 9	+	+	-	+
25[c]	206 ± 24	+	+	-	+ (5)[b]
37	80 ± 34	+	+	+	+ (1)[b]
Pre-treated at 37 °C					
10	0	-	-	-	-
25	5 ± 24	tr	tr	-	-
37	93 ± 8	-	-	-	-

[a] $n = 3 \pm$ SD, except where stated otherwise.

[b] Values in parenthesis indicates number of compounds present in extracts

[c] n = 2 ± SD

+, detected

-, not detected

tr, detected at trace level

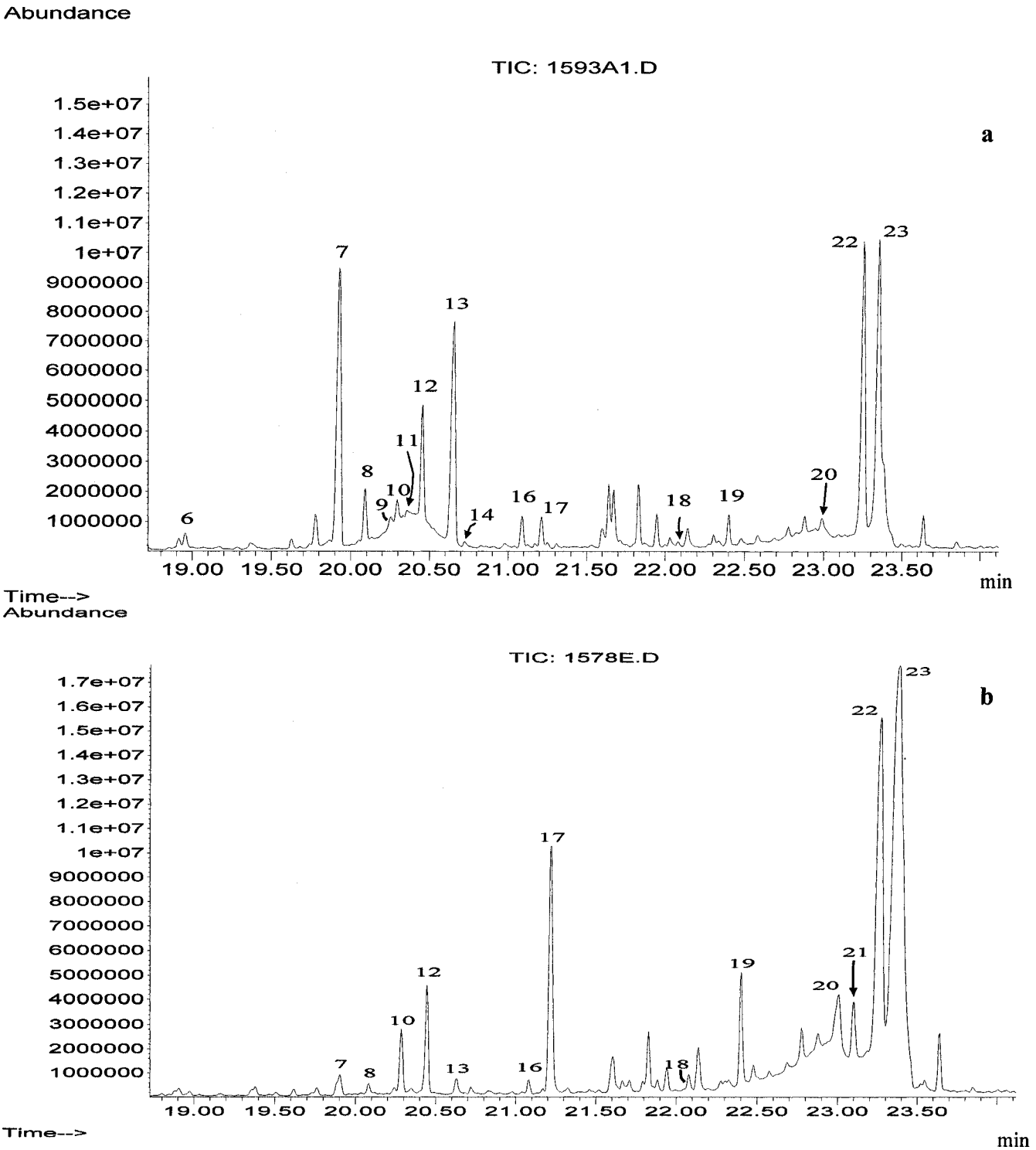


Fig. 4.1 A section of the TIC of acidic and neutral extracts of cultures of biphenyl-grown cells incubated with 2,4'-diCB at 10 °C for 4 h (a) and 48 h (b). See footnotes of Table 4.2 for likely structures for annotated peaks, and appendix C for the mass spectra.

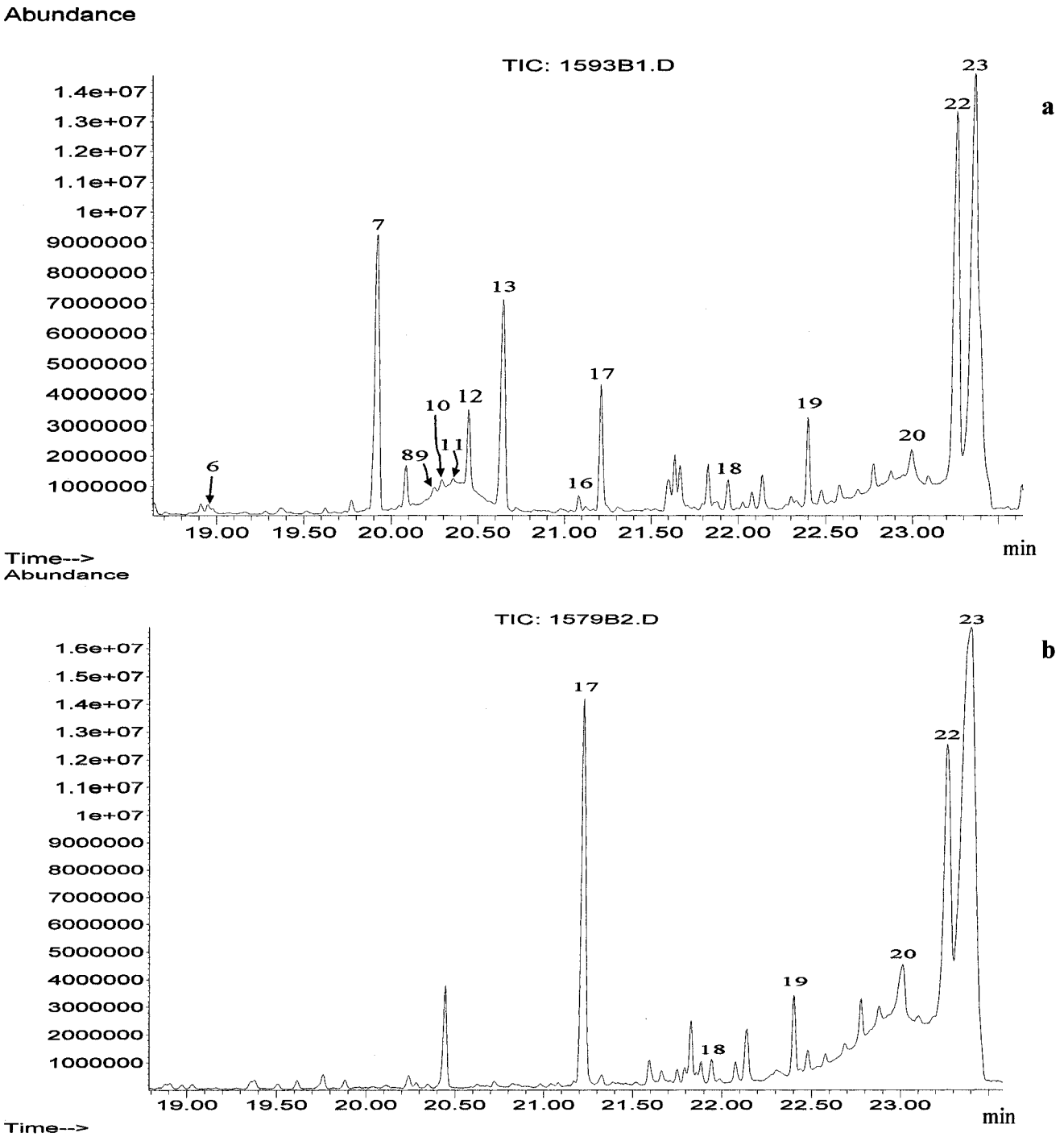


Fig. 4.2. A section of the TIC of acidic and neutral extracts of cultures of biphenyl-grown cells incubated with 2,4'-diCB at 25 °C for 4 h (a) and 48 h (b). See footnotes of Table 4.2 for likely structures for annotated peaks, and appendix C for the mass spectra.

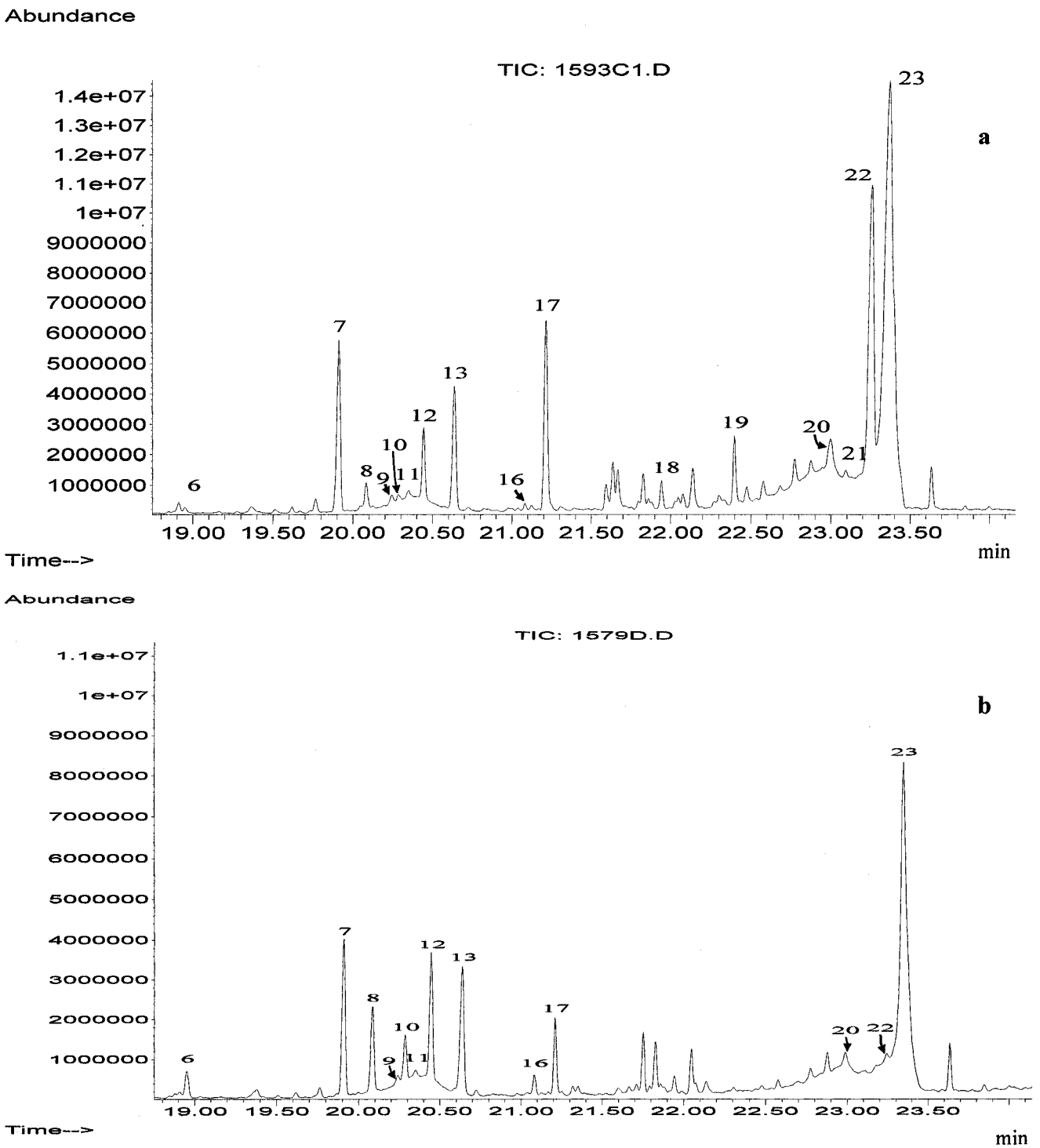


Fig. 4.3. A section of the TIC of acidic and neutral extracts of cultures of biphenyl-grown cells incubated with 2,4'-diCB at 37 °C for 4 h (a) and 48 h (b). See footnotes of Table 4.2 for likely structures of annotated peaks, and appendix C for the mass spectra.

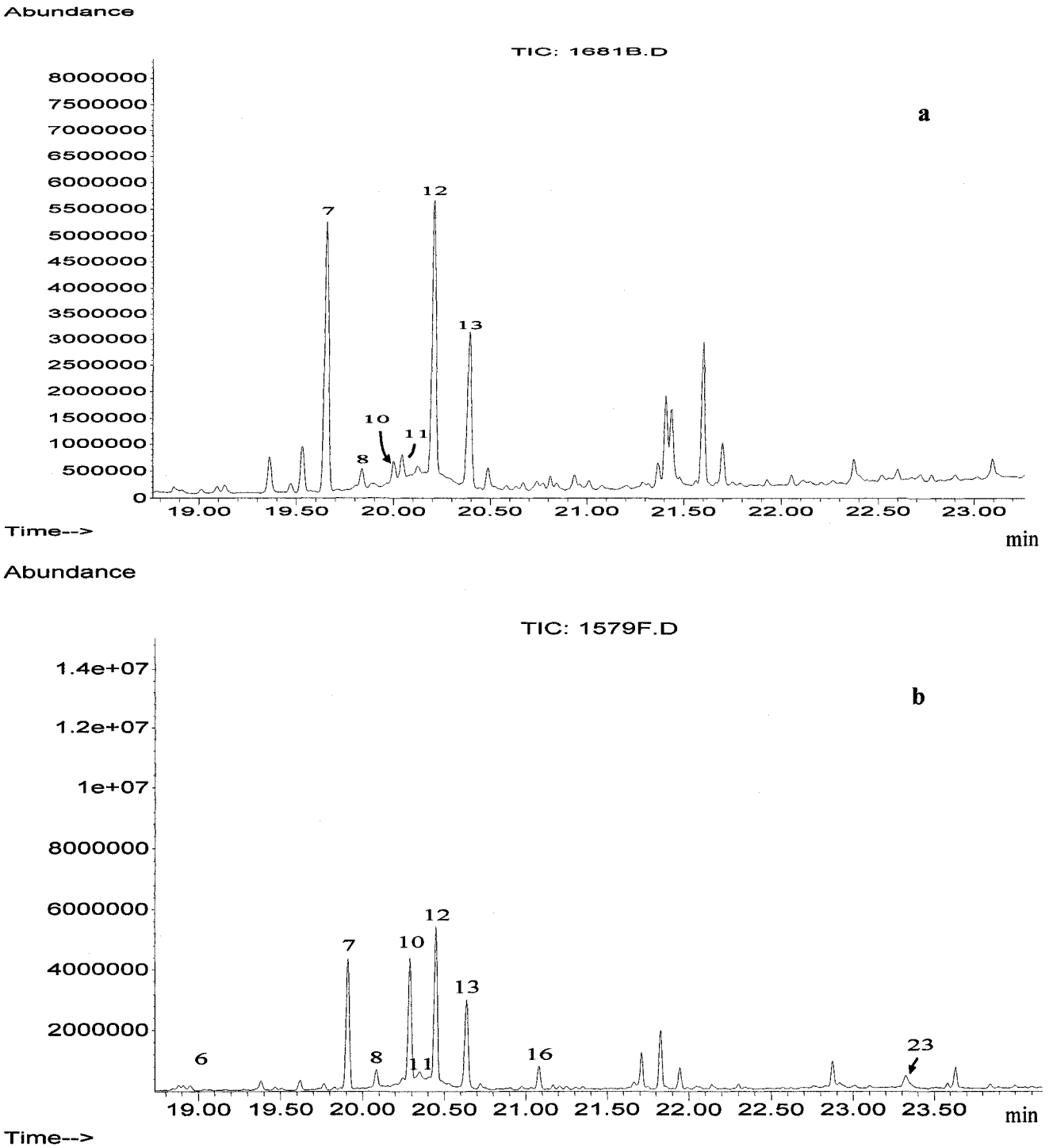


Fig. 4.4. A section of the TIC of acidic and neutral extracts of cultures of biphenyl-grown cells incubated with 2,4'-diCB at 45 °C for 12 h (a) and 48 h (b). See footnotes of Table 4.2 for likely structures for annotated peaks, and appendix C for the mass spectra.

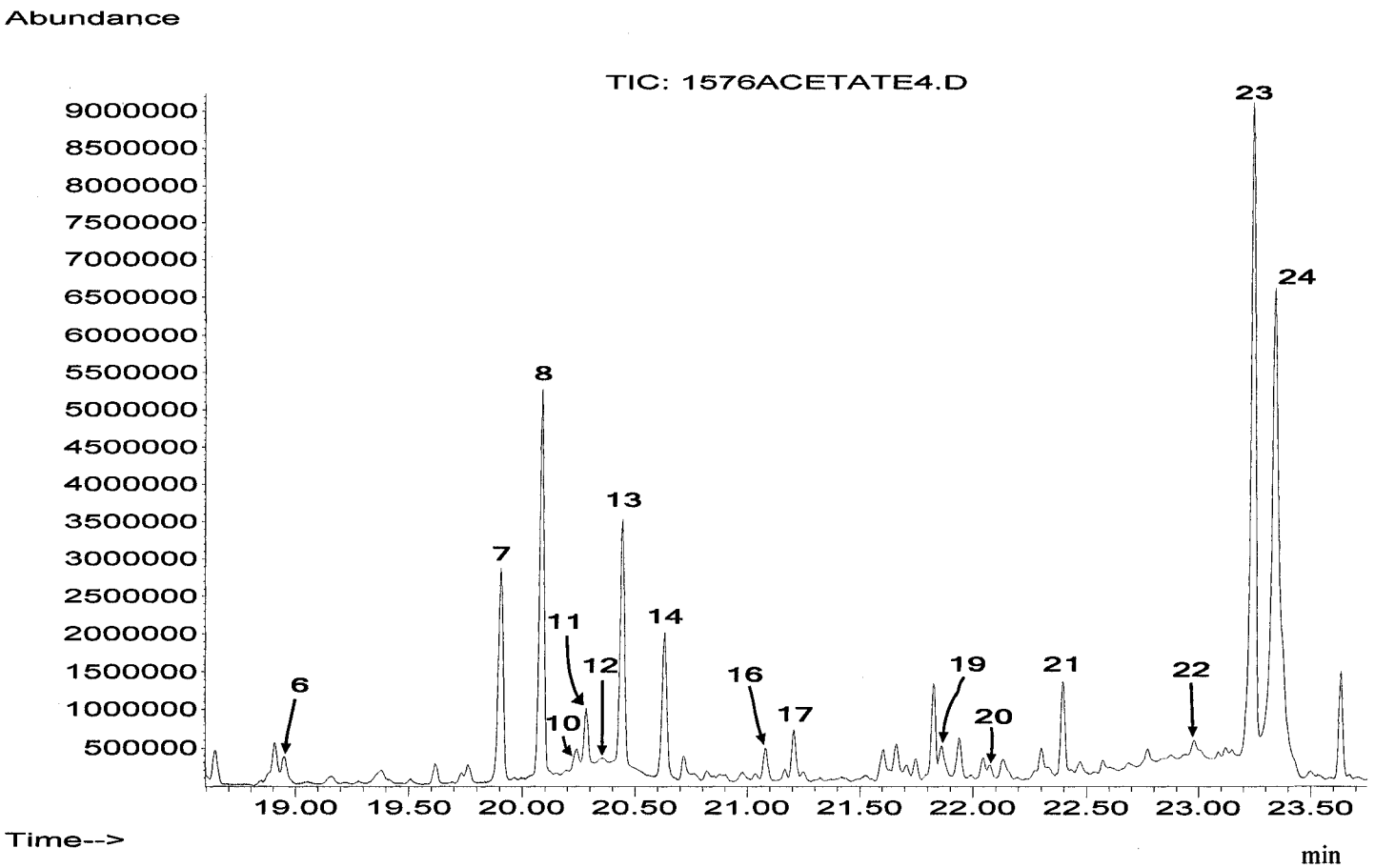


Fig. 4.5. A section of the TIC of acidic and neutral extracts of cultures of **acetate**-grown cells incubated with 2,4'-diCB at 10 °C for 48 h. Proposed structures of compounds in annotated peaks based on mass spectral data: peak nos. 6 and 8, dichloro-hydroxybiphenyls; peak nos. 7, 10, 11, 12, and 13, monochloro-dihydroxybiphenyl; peak no. 14, dichloro-dihydrodiols; peak nos. 16 and 19, dichloro-dihydroxybiphenyl; peak nos. 17, 20, 21, 22, 23, and 24, dichloro-HOPDAs. Note that peak nos. 15 (a dihydrodiol) and 18 (a dichloro-dihydroxybiphenyl) were not detected in the TIC above but they were present in other extracts. The spectra of the proposed compounds are similar to those detected in the extracts of cultures of pyruvate-, TSB-, or biphenyl-grown cells.

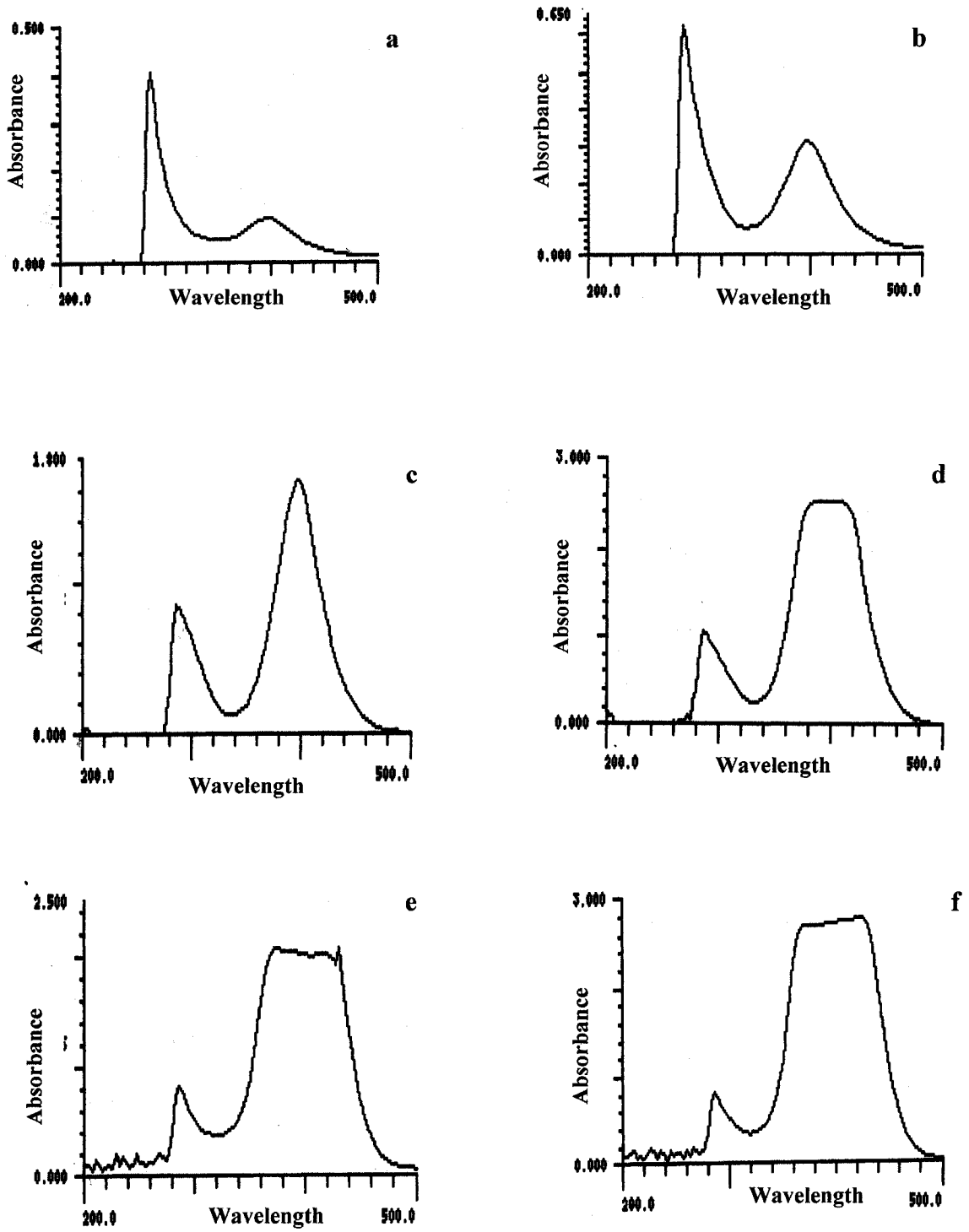


Fig. 4.6. Time-dependent accumulation of *ortho*-substituted HOPDA(s) from 250 μM of 2,4'-diCB by *Hydrogenophaga* sp. IA3 at 25 °C. Note incubation periods: a, 5 min; b, 30 min; c, 2 h; d, 4 h; e, 12 h; and f, 72 h .

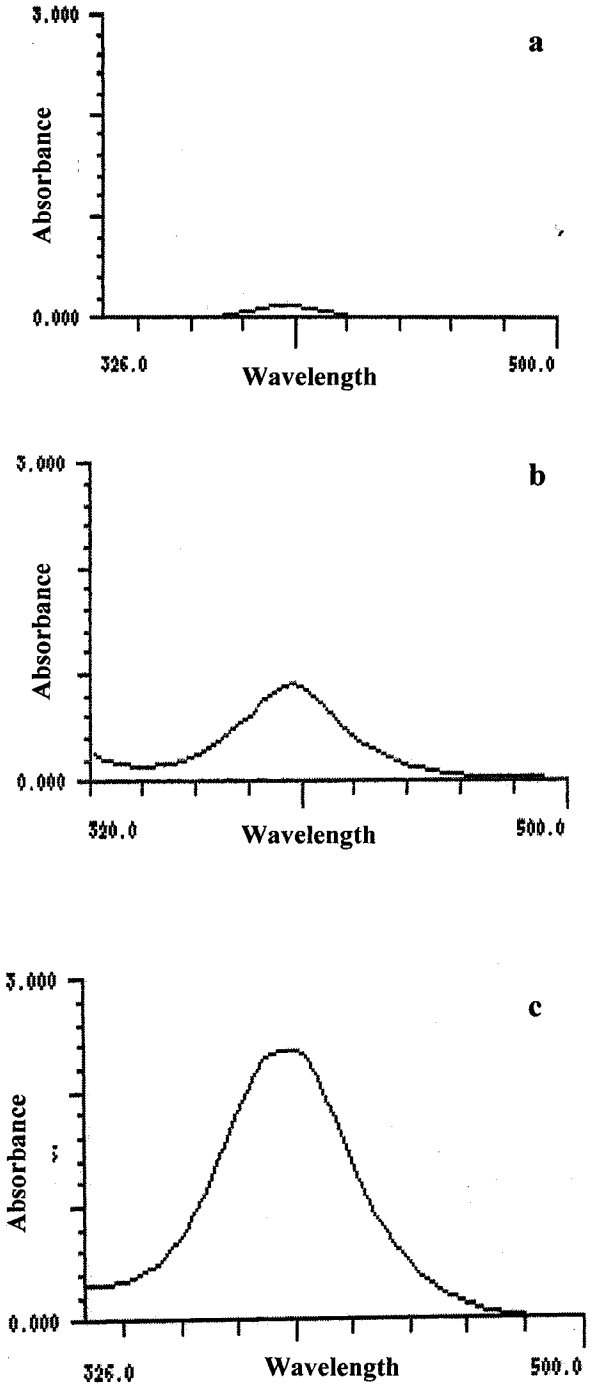


Fig. 4.7. Comparison of the level of HOPDA(s) in cultures that degraded 46 μM of 2,4'-diCB at 45 °C (a) and cultures that degraded 45 μM of 2,4'-diCB at 25 °C (b); c, cultures incubated with 250 μM of 2,4'-diCB at 45 °C for 5 min followed by incubation at 25 °C for 48 h.

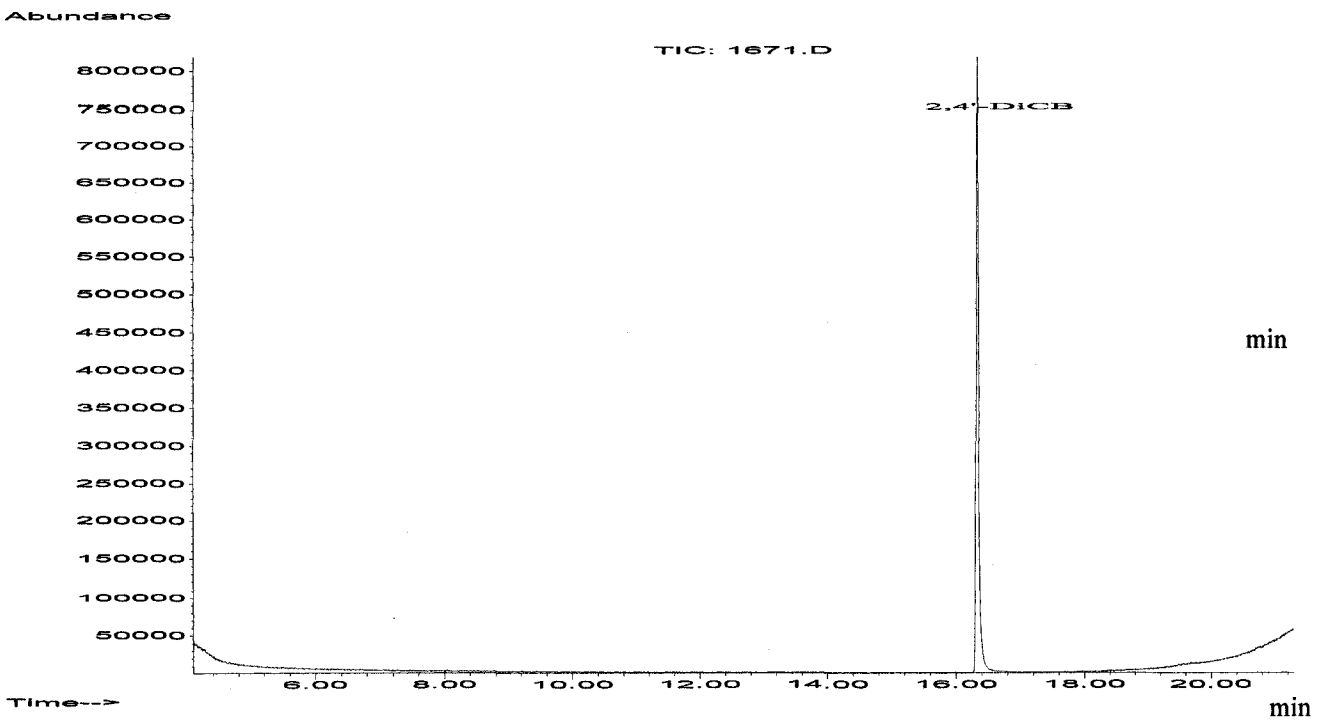


Fig. 4.8. TIC of acetone-stock solution of 2,4'-diCB showing the absence of contaminants.

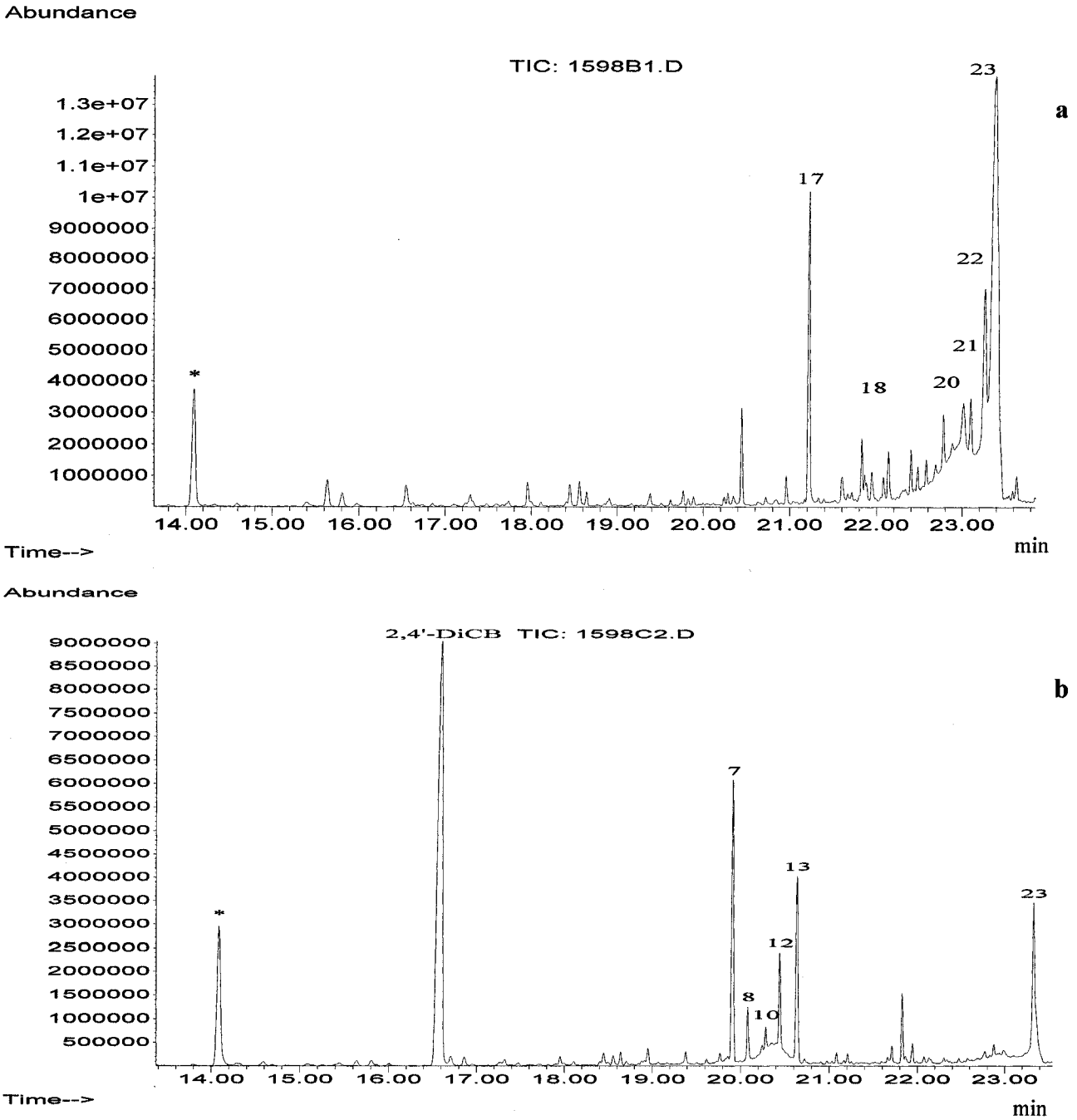


Fig. 4.9. A section of the TIC of extracts of cultures of biphenyl-grown cells incubated with 2,4'-diCB at 25 °C (a) and 37 °C (b) for 48 h after pre-treatment at 10 °C. Peak with asterisk denotes 2,3'-CBA added as a standard prior to derivatization. See footnotes of Table 4.2 for likely structures of annotated peaks, and appendix B for the mass spectra.

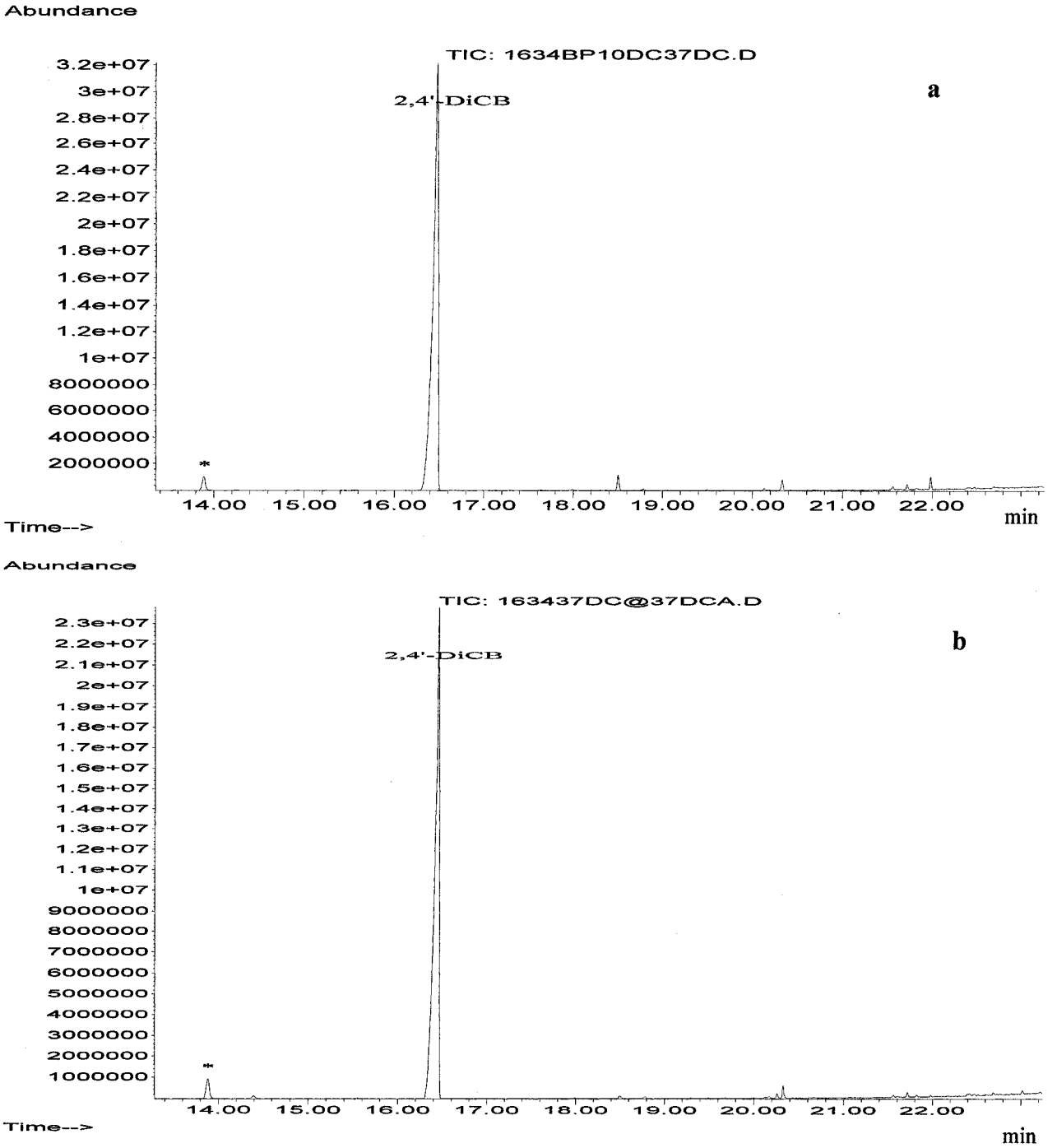


Fig. 4.10 A section of the TIC of extracts of cultures of biphenyl-grown cells incubated with 2,4'-diCB at 10 °C (a) and 37 °C (b) for 48 h after pre-treatment at 37 °C. Peak with asterisk denotes 2,3'-CBA added prior to derivatization as a standard. See footnotes of Table 4.2 for likely structures for annotated peaks.

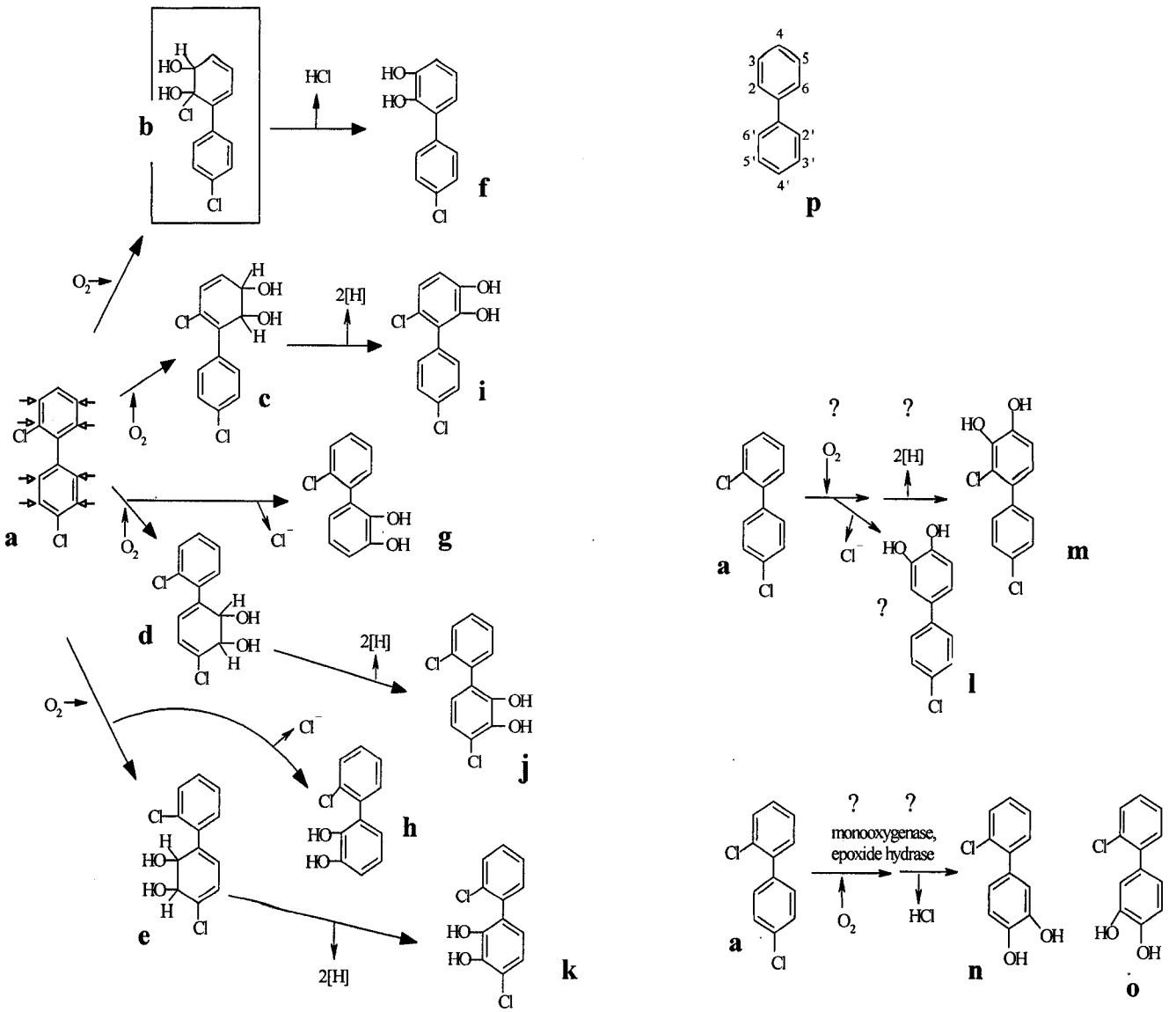


Fig. 4.11. Hypothetical routes of production of dihydroxylated compounds having similar structures as the intermediates that are formed from 2,4'-diCB by strain IA3-A. Note that the sites available for oxidation of parent compound (**a**) are indicated with open arrows. Question marks indicates lack of data to support the presence of the pathway in strain IA3-A. Compound labeled **c**, **d**, **e** are dihydrodiols (compounds in peak nos. 13, 14, and 15 in Fig. 4.1 – 4.4); **f**, **g**, **h**, **n** or **o**, and **l** are monochlorodihydroxybiphenyls (compounds in peak nos. 7, 9, 10, 11, 12 in Fig. 4.1 – 4.4); **i**, **j**, **k**, **m** are dichlorodihydroxybiphenyls (compound in peak no. 16 in Fig. 4.1 – 4.4). See appendix B for mass spectrum of TMS-derivatives of the compounds shown above. Note: two dihydrodiols were detected using acetate-grown cells.

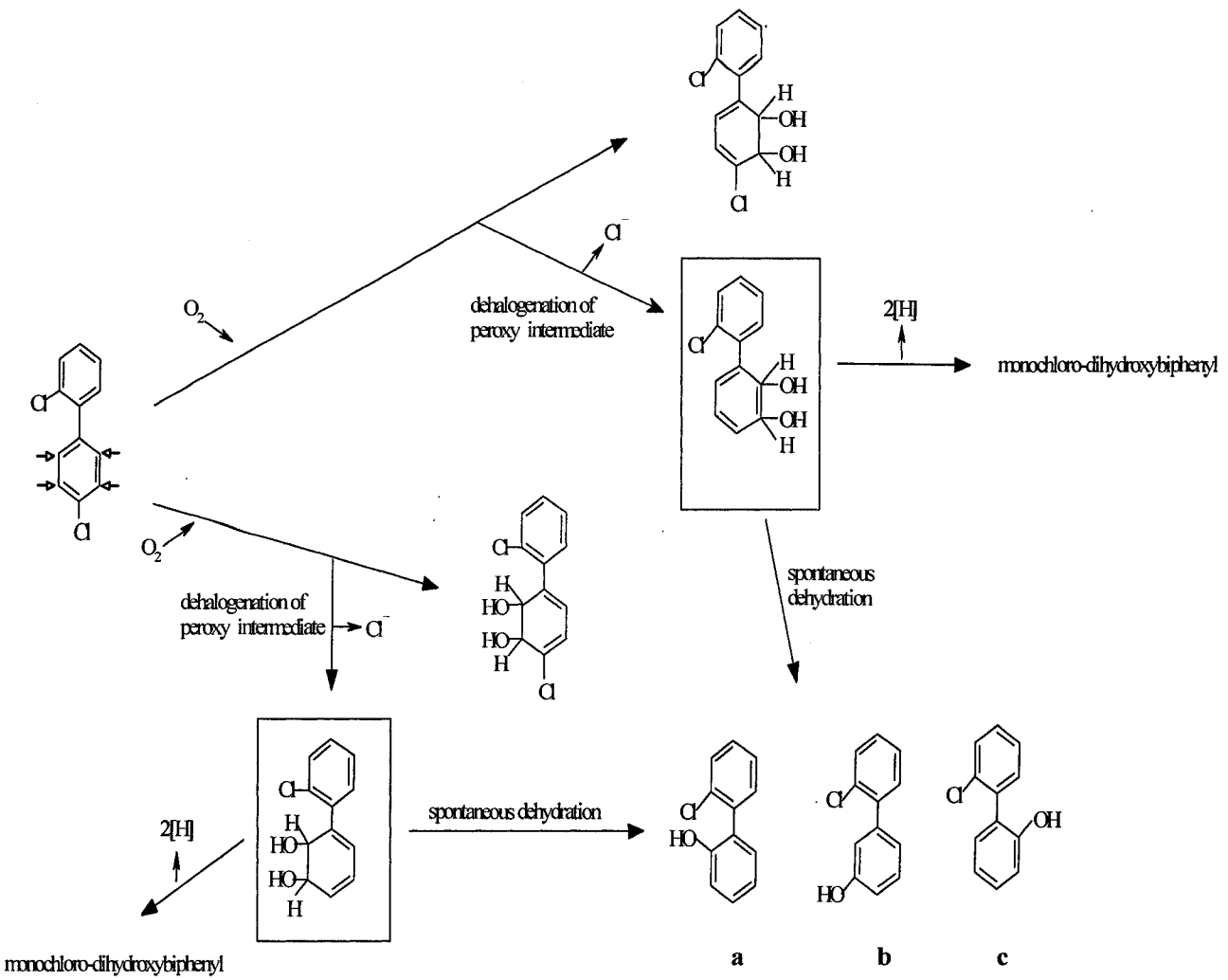


Fig. 4.12. Hypothetical routes leading to formation of monochlorohydroxybiphenyls (compounds labelled **a**, **b**, **c**; which are compounds in peak nos. 3, 4, 5 in Fig. 4.1-4.4). Note that evidence for similar side reactions during transformation of 2,4'-diCB was proposed previously by Ahmad *et al.* (1991). Conformational flexibility of compounds **a** and **c** may permit elution at different retention times on GC. See appendix B for complete mass spectra of the compounds.

4.4. Discussion

Significant degradation of 2,4'-diCB at low temperature by *Hydrogenophaga* sp. IA3-A is not surprising. Psychrotolerant organisms produce cold-adapted enzymes that have flexible structure, lower thermostability, and higher catalysis efficiency at low temperatures (Feller and Gerday, 1997). Poor degradation of 2,4'-diCB at 45 °C may be due to sensitivity of 2,4'-diCB-degrading enzymes to high temperature or inhibition of cellular functions that are relevant to metabolism of 2,4'-diCB. Strain IA3-A was active against 2,4'-diCB at 5 °C (Chapter 3; Lambo and Patel, 2006a). There are no reports of degradation of pure 2,4'-diCB at low temperatures by other PCB-degrading psychrotolerant bacteria, but degradation of 2,4'-diCB in commercial mixtures of PCBs at low temperature has been reported before (Master and Mohn, 1998). Degradation of PCBs in Aroclor 1242 was sensitive to high temperature (Master and Mohn, 1998). Strain IA3-A grew on tryptic soy broth at 40 °C, but it did not grow at 45 °C (Chapter 2; Lambo and Patel, 2006b). Strain IA3-A also grew on biphenyl at 5 ° to 35 °C (Chapter 2; Lambo and Patel, 2006a). Thus, cells were expected to retain significant activity against 2,4'-DiCB at 37 °C (Table 4.1).

The effect of low temperature on the water solubility of organic compounds dictates that the amount of 2,4'-diCB that is available for degradation at 10 ° or 25 °C would be lower when compared to the amount available at higher temperatures. However, the similar extents of removal of 2,4'-diCB at 10 °, 25 °, and 37 °C showed that factors other than solubility of the compounds determines the removal of the congener at these temperatures. The addition of the compound into cell suspension as a dissolved stock solution of acetone also suggest that the removal of the congener in the aqueous phase is also governed by the rate of dissolution of the congener from acetone into the aqueous phase.

The production of CBAs at 10 °, 25 °, and 37 °C was much lower when compared to the production of CBAs at 45 °C (Table 4.1). The recovery of 2,4'-diCB as CBAs and extents of accumulation of HOPDAs at 10 °, 25 °, and 37 °C (Table 4.1) suggests that the pattern of transformation of 2,4'-diCB at low or moderate temperatures by strain IA3-A is comparable to the pattern of transformation of 2,4'-diCB by mesophilic *R. erythreus* NY05 and *Comamonas testosteroni* VP44 (Maltseva *et al.*, 1999) when most of the degraded 2,4'-diCB was recovered as HOPDA. It is important to note that only one HOPDA compound was detected by GC in those studies (Maltseva *et al.*, 1999). The recovery of the degraded 2,4'-diCB as CBAs at 45 °C suggests that the pattern of transformation of 2,4'-diCB at 45 °C is comparable to the pattern of transformation of 2,4'-diCB by mesophilic *Rhodococcus* sp. RHA1 and *Burkholderia xenovorans* LB400, where 97 and 87 % of the congener was recovered as 4-CBA, respectively (Maltseva *et al.*, 1999). Strain RHA1 and LB400 did not accumulate HOPDAs from 2,4'-diCB (Maltseva *et al.*, 1999). The extent of degradation of 2,4'-diCB was about six fold less at 45 °C compared to lower temperatures. Low level of HOPDA and recovery of 78 % of the degraded 2,4'-diCB as 4-CBA at 45 °C (Table 4.1) suggests that the pattern of transformation of 2,4'-diCB was different at 45 °C. In addition, cells incubated with 45 μM of 2,4'-diCB at 10 ° or 25 °C for 24 h accumulated relatively high level of HOPDAs. This observation also support the conclusion that the pattern of transformation of 2,4'-diCB was different at 45 °C, since transformation of 46 μM of 2,4'-diCB at 45 °C (Table 4.1) did not result in high level of HOPDAs. However, there is no evidence at this point that suggest that differences in the production of CBAs or accumulation of HOPDAs is directly related to the regiospecificity of cells of strain IA3-A

for 2,4'-diCB at different temperatures. Such evidence, in future studies, would require the purification of biphenyl-2,3-dioxygenase enzyme from the cells of strain IA3-A.

The detection of several isomeric intermediates in cultures incubated with 2,4'-diCB or 2,4,4'-triCB suggests that multiple pathways might be involved in the transformation of PCBs in strain IA3-A (Table 4.2 and Table 4.3). Though, it was reported previously that hydroxylation of a chloro-substituted ring may lead to artifactual dechlorination during derivatization or GC-MS analysis (Seeger *et al.*, 1995), but the likelihood that this kind of reaction resulted in the production of the mono- or dichloro-hydroxybiphenyls detected here is unlikely. The fact that several isomers of different intermediates were detected in cultures incubated with 2,4'-diCB, as well as 2,4,4'-triCB limits the possibility that the hydroxylated intermediates were exclusively produced during derivatization. Most of the intermediates detected in the extracts of cultures incubated with 2,4'-diCB are similar to the intermediates that are formed from 2,4'-diCB by some mesophilic strains (Furukawa *et al.*, 1979a; Ahmad *et al.*, 1991; Maltseva *et al.*, 1999). But unlike these strains, several isomeric products were formed from 2,4'-diCB by strain IA3-A. The hypothetical routes leading to the formation of many of these hydroxylated intermediates is presented in Fig. 4.11 and 4.12. The formation of isomeric HOPDAs from 2,4'-diCB in cultures incubated at low or moderate temperatures is unusual. It is likely that the formation of HOPDAs from chlorobiphenyls is strain dependent. Masse *et al.* (1989) reported the production of three HOPDAs from 4-chlorobiphenyl, and the authors proposed two mechanisms of formation of the isomers in culture broths. It is possible that similar mechanisms caused the formation of some of the HOPDAs detected here. It is important to emphasize that only one HOPDA compound was detected in the extracts of cultures incubated at 45 ^{o}C. The available data suggests that there

are different pathways for the transformation of 2,4'-diCB in strain IA3-A and that it is possible that these pathways were regulated differently at different temperatures. It is suspected that low or moderate temperatures favoured the predominance of transformation routes that caused excess level of HOPDAs. The temperature-dependence of the conformation and activity of cold-adapted enzymes are well known (Patel and Bartlett, 1988; Feller, 2003). It could be that certain enzymes involved in the degradation of 2,4'-diCB were activated at 45 °C, resulting in faster conversion of some intermediates. This may account for the low level of HOPDAs at this temperature. It can be expected that one of such enzymes might include HOPDA-hydrolase, the enzyme responsible for the conversion of HOPDAs to CBAs. Further studies with purified enzymes preparation are required to confirm these.

Additional studies using cells pre-treated at different temperatures showed that temperature influences the pattern of transformation of 2,4'-diCB, because cells pre-treated at 10 °C generally retained the ability to degrade 2,4'-diCB, as well as the ability to form metabolic intermediates from the compound (Table 4.4). In contrast to this, cells pre-treated at 37 °C either failed to degrade 2,4'-diCB or they lost the ability to form extractable intermediates from it.

One of the challenges of using microorganisms for the biological treatment of PCB-contaminated sites is the production and accumulation of toxic products. These products could be formed from *ortho-* plus *para-* substituted chlorobiphenyls (Bedard and Haberl, 1990; Maltseva *et al.*, 1999). These PCBs are candidates for the aerobic phase of the sequential anaerobic-aerobic process for decontamination of PCBs, which is the most promising option for biological treatment of PCB-contaminated sites. The current study suggests that excess accumulation of problematic HOPDAs from a model *ortho-* plus *para-*

subtituted congener could be avoided at a specific temperature. However, a thorough understanding of the mechanism behind the observations reported here would require additional studies. These studies should include purification of individual biphenyl-degrading enzymes from cells of strain IA3-A and investigation of the effect of temperature on the pattern of transformation of their respective substrates.

Chapter 5

Some Properties of Partially Purified Cold-Adapted Biphenyl-2,3-Dioxygenase from *Hydrogenophaga* sp. IA3-A and Investigation of its Role in the Catabolism of 2,4'-Dichlorobiphenyl

5.1 Introduction

Oxygenases catalyze the incorporation of oxygen into different classes of compounds and they are involved in the degradation of numerous aromatic substrates through biosynthetic pathways that are used for dissimilation or co-metabolic catabolism of the substrates. The major classes of oxygenases include monooxygenases that catalyze the incorporation of one atom of oxygen into their substrates, and dioxygenases that catalyze the incorporation of two atoms of oxygen into their respective substrates (Harayama *et al.*, 1992).

There are two types of dioxygenases; these are aromatic-ring dioxygenases and aromatic-ring-cleavage dioxygenases. The aromatic-ring dioxygenases use NAD(P)H as electron donor and they incorporate two hydroxyl groups into aromatic substrates to form *cis*-diols. Aromatic-ring-cleavage dioxygenases incorporate two atoms of dioxygen into aromatic substrates, and the aromatic ring is cleaved. The latter reaction does not require an external reductant (Harayama *et al.*, 1992). Bacterial dioxygenases are three component enzymes consisting of a terminal dioxygenase and a short electron transport chain.

The four-step aerobic enzymatic conversion of biphenyl and polychlorinated biphenyls (PCBs) into, respectively, benzoate and chlorinated benzoates is widespread in

many bacterial isolates (Seeger *et al.*, 1995; Khan and Walia, 1989; Bedard *et al.*, 1986; Furukawa *et al.*, 1979a; Ahmed and Focht, 1973). The first reaction of the biphenyl catabolic pathway (bph pathway) is catalyzed by a aryl hydroxylating dioxygenase, biphenyl-2,3-dioxygenase (BPDO), that inserts two atoms of oxygen into one of the aromatic rings. BPDO utilize oxygen and electrons from NAD(P)H to convert biphenyl to *cis*-2,3-dihydroxy-1-phenylcyclohexa-4,6-diene or 2,3-dihydro-2,3-dihydroxybiphenyl (Haddock and Gibson, 1995; Gibson *et al.*, 1973). BPDO is a multicomponent protein that is composed of a terminal two-subunit oxygenase with a $\alpha_3\beta_3$ constitution, a ferredoxin, and a FAD-dependent reductase. The oxygenase contains a Rieske-type [2Fe-2S] cluster and a mononuclear iron center, while the ferredoxin only contains a 2Fe-2S center (Haddock and Gibson, 1995; Hurtubise *et al.*, 1995). Studies suggest that the ferredoxin and reductase components form a short electron-transport chain that transfer electrons from NAD(P)H to the oxygenase components that contains the active site for incorporation of oxygen into aromatic substrate (Haddock *et al.*, 1993).

To date, the BPDO that have been characterized were obtained from mesophilic strains (Imbeault *et al.*, 2000; Chebrou *et al.*, 1999; Haddock and Gibson, 1995; Haddock *et al.*, 1993). In previous chapter, evidence was presented that temperature determines the pattern of transformation of 2,4'-diCB by *Hydrogenophaga* sp. IA3-A, and preliminary evidence was also presented indicating that multiple pathways were involved in the metabolism of 2,4'-diCB. In the present chapter, BPDO from biphenyl-grown cells of *Hydrogenophaga* sp. IA3-A would be partially purified and characterized under different conditions. Due to limited resources at this point of the research, BPDO could not be investigated for its role in the temperature-dependent transformation of 2,4'-diCB.

5.2 Materials and Methods

5.2.1 ***Culture conditions and preparation of cell extracts.*** *Hydrogenophaga* sp. IA3-A was isolated from PCB-contaminated soils following the procedures outlined in chapter two. Cells were grown on 0.5 % (w/v) of biphenyl in 1 L of mineral salt medium (MSM) composed of (g L^{-1}): $(NH_4)_2SO_4$ (0.5), $MgSO_4.7H_2O$ (0.1), $Ca(NO_3)_2$ (0.075), plus vitamins (1 mL) and trace elements solution (10 mL) per litre of 40 mM phosphate buffer (KH_2PO_4 and Na_2HPO_4, pH 7.3) (Adriaens *et al.*, 1989). The composition of the vitamins and trace elements solution was the same as those described in chapter two. Cultures were incubated at 30 °C at 200 rpm and growth was monitored by measuring optical density at 600 nm using a Shimadzu spectrophotometer. Cells were harvested at mid-late exponential phase by centrifugation (6,000 rpm at 4 °C for 20 min). Harvested cell pellets were washed twice in 0.05 M sodium phosphate buffer (pH 7.5) and stored at -20 C prior to preparation of cell extracts.

Cells (80 g) were thawed and washed in 50 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid buffer (HEPES) (pH 7.3) containing 10 % (v/v) glycerol. Cells were suspended in 160 mL of HEPES (pH 7.3) containing 10 % (v/v) glycerol, 1 mM dithiotrietol, 0.2 mM phenylmethylsulfonyl, 0.01 g L^{-1} each of DNase I and RNase A. The cell suspension was passed twice through a chilled French pressure cell, and the suspension was centrifuged (145,000 *g* at 4 °C for 1 h) using an Optima L-90 K ultracentrifuge (Beckman Coulter Inc., CA, USA). The clear supernatant and pelleted membrane fractions were collected separately and stored at -70 °C.

5.2.2 ***Partial purification of biphenyl-2,3-dioxygenase.*** Seventy millilitre of the crude extract was diluted with equal volume of chilled distilled water containing 10 % (v/v) glycerol to achieve a final concentration of 25 mM HEPES (pH 7.3) plus 10 % (v/v) glycerol. The diluted extract was applied at a flow rate of 2.2 mL min^{-1} to a Fast-Flow DEAE-Sepharose column (2.6 cm by 30 cm; Pharmacia LKB Biotechnology) that was equilibrated with 25 mM HEPES (pH 7.3) containing 10 % (v/v) glycerol. The column was washed with 300 mL of the same buffer, followed by a linear salt gradient from the starting buffer to 0.4 M NaCl (1400 mL). Thereafter, a second gradient of 0.4 to 2.0 M NaCl (300 mL) was applied to the column. Alternatively, remaining portion of the crude extract (60 mL) was diluted with equal volume of distilled water containing 10 % (v/v) glycerol to achieve a final concentration of 25 mM HEPES (pH 7.3) plus 10 % (v/v) glycerol. This portion was loaded separately onto a Fast-Flow DEAE-Sepharose column (2.6 cm by 30 cm; Pharmacia) that was washed using similar procedures as above except that bound protein was eluted using a linear salt gradient from the starting buffer to 0.3 M NaCl, followed by a second gradient of 0.3 to 2.0 M NaCl.

Fractions from each of the major DEAE-Sepharose chromatography peaks were pooled and amended with 0.55 g mL^{-1} of ammonium sulphate. Precipitated protein was collected by centrifugation, and redissolved and dialyzed in 10 mM potassium phosphate buffer (pH 7.0) containing 2 mM dithiotrietol and 10 % (v/v) glycerol or in 25 mM 2-(*N*-morpholino)ethanesulfonic acid (MES) buffer (pH 6.0) containing 1.0 mM dithiotrietol, 1 mM EDTA, 0.2 mM phenylmethylsulfonyl, and 10 % (v/v) glycerol. Dialysis was carried out for 12 h in 4 – 8 L of each buffer. Fast-Flow DEAE-Sepharose chromatography separated the BPDO of strain IA3-A into three components that were required together

for the oxidation of biphenyl. A yellow colour fraction (fraction A) contained the reductase component, a second fraction (fraction E) that was brownish in colour with absorbances at 323 nm and 455 nm typical of a Rieske-type protein contained the oxygenase component, while a third fraction (fraction F) that was also brownish in colour contained the ferredoxin component. The oxygenase and ferredoxin component were purified further, while the reductase component was used without further purification.

Initial attempt to purify the oxygenase component was carried out using Octyl-Sepharose CL-4B. The sample (15 mL) was adjusted to 1.5 M $(NH_4)_2SO_4$, and it was then centrifuged (1500 rpm at 5 ^{o}C for 30 min). The supernatant was applied at a flow rate of 1.0 mL min^{-1} to an Octyl-Sepharose CL-4B column (1.5 cm by 12 cm) that was equilibrated with 25 mM MES buffer (pH 6.0) containing 10 % glycerol and 1.5 M NH_4SO_4. Unbound protein was eluted with the equilibration buffer (425 mL), and bound protein was released with a decreasing salt gradient from 1.5 to 0.0 M $(NH_4)_2SO_4$ dissolved in 25 mM MES buffer (pH 6.0) containing 10 % glycerol (500 mL). The column was washed further with equilibration buffer (170 mL), equilibration buffer plus 50 % (v/v) ethylene glycol (360 mL), and then equilibration buffer plus 30 % (v/v) isopropanol (400 mL). Attempt to release most of the bound protein using the procedures described above was not successful.

A second attempt to further purify the oxygenase component was carried out using a hydroxylapatite column that was equilibrated with 10 mM potassium phosphate (pH 7.0). The sample was loaded onto the column (2.6 x 27 cm; Pharmacia) at a flow rate of 0.2 mL min^{-1} and the column was washed with 100 mM potassium phosphate (50 mL, pH 7.0). Bound protein was eluted with 250 mM potassium phosphate (400 mL) (pH 7.0) at a

flow rate of 0.23 ml min^{-1}. Fractions (73 ml) with characteristic absorbance at 323 and 455 nm typical of proteins with Rieske-type iron-sulphur center were collected. The pooled fractions were amended with 0.55 g ml^{-1} of $(NH_4)_2SO_4$ and precipitated protein was collected by centrifugation and redissolved in 4 ml of 10 mM potassium phosphate (pH 7.0). Protein was desalted for 12 h in 5 L of 10 mM potassium phosphate (pH 7.0) containing 10 % (v/v) glycerol and 2 mM dithiotreitol. The dialysate (7 ml) was centrifuged to remove any precipitate prior to storage at -70 °C. The ferredoxin component was also purified further using the procedures described above except that bound proteins in the hydroxylapatite column (2.6 x 30 cm; Pharmacia) was eluted with 100 ml of 100 mM potassium phosphate (pH 7.0) and 250 ml of 250 mM potassium phosphate (pH 7.0). Protein were precipitated and desalted as described above, and the dialysate (7.1 ml) was stored at -70 C.

5.2.3 ***Analytical methods.*** Biphenyl 2,3-dioxygenase activity in membrane fractions, crude extracts and fractions from Fast-Flow DEAE-Sepharose and hydroxylapatite column was performed at 30 °C by following oxygen consumption. The reactions were carried out using a Clark-type oxygen electrode (Yellow Springs Instrument Co., OH, USA) connected to a reaction chamber containing 2 ml of air-saturated 50 mM MES (pH 6.0). The assay contained 500 μM NADH or 400 μM NADPH, 250 μM biphenyl, 200 μM $Fe(SO_4)_2(NH_4)_2$ and partially purified fractions. Reactions were initiated by addition of biphenyl after equilibrating the mixtures for 20 – 30 s. Activity was corrected for oxygen uptake in separate reactions containing NADH or NADPH, $Fe(SO_4)_2(NH_4)_2$, and

partially purified fractions except biphenyl. One unit of enzyme activity is defined as 1 μmol of O_2 consumed per minute.

The standard assay (750 μL total volume) for characterization of BPDO was carried out at 30 °C in air-saturated 50 mM MES buffer (pH 6.0). The reaction mixture contained 100 or 166.66 μM biphenyl, 33.33 μM NADH, 6.8 μM $Fe(SO_4)_2(NH_4)_2$, 1.0 μM FAD and each of the partially purified components. The concentration of biphenyl, NADH, FAD, and protein were optimized in order to obtain a linear reaction. The partially purified components were added separately into the mixtures, and reactions were initiated by adding biphenyl already dissolve in 1 μL of *N*-dimethylformamide. The reactions were carried out in 1 ml glass cuvette and incubated for 2 min at 30 °C. The decrease in absorbance at 340 nm was used to monitor NADH oxidation immediately after the incubation period. Similar reactions mixture containing all of the components except the substrate was used as a control. Specific activity is defined as nmol of NADH oxidized per min per milligram protein.

Physical parameters such as pH (5.0–7.0), substrate concentration (0 – 500 μM), temperature (5 ° – 55 °C), FAD concentration (0 – 40 μM) were investigated for their effects on BPDO activity. The effects of various aromatic substrates on BPDO activity were determined at 30 °C, and the effects of selected aromatic substrates on BPDO activity were also examined at 7 °C. The effects of various solvents including Triton X-100, Tween 80, and acetone, and EDTA on BPDO activity were also examined. The reaction mixture contained 166.66 μM biphenyl, 33.33 μM NADH, 6.8 μM $Fe(SO_4)_2(NH_4)_2$, 0 or 1.0 μM FAD and partially purified components in air-saturated 50 mM MES buffer (pH 6.0).

The effects of metals like cobalt, zinc, mercury, nickel, molybdate, ferric ion, copper, manganese, and magnesium on BPDO activity were examined. The thermostability of BPDO was also determined by incubating the partially purified components at various temperatures (0 – 65 ^{o}C) in 50 mM MES (pH 6.0) for 2 min. In addition, optimal substrate concentration (0 – 25 μM) was determined in 0.05 M sodium phosphate (pH 7.0) buffer, and this was compared to results obtained using 50 mM MES (pH 6.0) buffer. In these cases, except stated otherwise, reaction mixtures contained 166.66 μM biphenyl, 33.33 μM NADH, 6.8 μM $Fe(SO_4)_2(NH_4)_2$, 1.0 μM FAD and partially purified components in air-saturated 50 mM MES buffer (pH 6.0).

5.3 Results

5.3.1 ***Partial purification of biphenyl dioxygenase.*** After the release of lysate of biphenyl-grown cells of *Hydrogenophaga* sp. IA3-A, BPDO activity was detectable in the soluble fraction (Table 5.1). The membrane fraction did not contain BPDO activity. In addition, the soluble fraction exhibited relatively high activity when incubated at 10 ^{o}C. Fractionation of cell extract on a Fast-Flow DEAE-Sepharose column yielded several protein fractions (Fig. 5.1 and 5.2) and three of these fractions (A, E, and F) were required together for maximum activity (Table 5.1). One of the fractions that eluted at lower salt concentration was yellowish in colour and it was designated as a component A. Another fraction was brownish in colour and it exhibited characteristic absorbance at 323 and 455 nm typical of Rieske-type iron-sulphur protein was designated as component E. The third fraction that eluted toward the end of the linear salt gradient was also brownish in colour and it was designated as component F. Component A and F stimulated BPDO activity in cell extracts and the effect was higher when the two were present together (Table 5.1).

Initial attempt to further purify fraction E using an Octyl-Sepharose CL-4B column was not successful and most of the bound protein could not be eluted after washing the column with equilibration buffer containing ethylene glycol or isopropanol (Fig. 5.3). However fraction E and fraction F were further purified using hydroxylapatite column (Fig. 5.4 and 5.5). The duration of this purification step was likely responsible for the slight loss in activity (Table 5.2). The details of the purification procedures are summarized in Table 5.2.

5.3.2 ***Characterization of partially purified BPDO.*** BPDO exhibited high activity against 4-monoCB, and activity against biphenyl, 2-monoCB, and 2,4'-diCB were generally similar (Table 5.3). BPDO activity was very low in the presence of trichlorinated biphenyls, 2,2'4- and 2,4,4'-triCB. Unlike benzoate, chlorobenzoates (CBAs) stimulated BPDO activity, while substituted and unsubstituted catecholic substrates did not stimulate BPDO activity. When FAD was included in the reaction mixtures, BPDO activity was only stimulated at lower concentrations of the cofactor (Table 5.4). Except for benzene, and to some extent 4-monoCB, incubation of the enzyme at low temperature with some aromatic substrates did not severely affect BPDO activity (Table 5.5).

BPDO was thermostable at temperatures between 0 – 63 °C, and activity was only not detected when BPDO was stored at 65 °C (Table 5.6). In addition, cells transformed 2,4'-diCB at 55 °C to hydroxylated intermediates and CBAs (Fig. 5.6, Fig. 5.7). This suggests that BPDO and other enzymes of the upper pathway for (chloro)biphenyl metabolism were active at high temperatures. EDTA, and solvents such as Triton X-100, Tween 80, and acetone inhibited BPDO activity (Table 5.7). Metal ions that were tested suppressed BPDO activity to different extent (Table 5.7), but the effect of metal ions such as mercury and copper on BPDO activity was severe. The optimal pH, temperature, and substrate concentration for BPDO activity in 50 mM MES buffer was 6.0, 25 °C, and 150 μM, respectively (Fig. 5.8, Fig. 5.9, and Fig. 5.10). The apparent K_m of partially purified BPDO for biphenyl was 77.5 µmol and the apparent V_{max} value for NADH was 0.04 μmol per minute per milligram protein.

Table 5.1 Oxygen consumption by cell membrane and cell extracts, and requirement for biphenyl dioxygenase activity in DEAE-Sepharose chromatographic fractions of crude extracts of *Hydrogenophaga* sp. IA3-A

Chromatographic fractions	Activity[a] (nmol)
A + B + E	0.05
A + B + F	0.21
A + C + E	0.17
A + C + F	0
A + D + E	0
A + D + F	0
A + E + F	0.50
A + F	0
B + E + F	0.11
A + Cell extract[b]	0.78
A + F + Cell extract[b]	2.31
F + Cell extract[b]	0.64
Cell extract[b]	0.62 (0.41)[c]
Cell membrane[b]	0

[a]Oxygen consumption at 30 °C (Values are mean of duplicate reactions).

[b]NADPH present as a reductant; plus 100 μL of cell extract or membrane fraction

[c]Value in parenthesis indicates oxygen consumption at 10 °C

Composition of reaction mixtures in 2 ml of 50 mM MES buffer (pH 6.0): 250 μM biphenyl; 500 μM NADH or 400 μM NADPH; 200 μM $Fe(SO_4)_2(NH_4)_2$, and 50 μL of component A, B, D, E, F; 75 μL of component C; and 25 μL of cell extract.

Table 5.2. Partial purification of oxygenase component from cells of *Hydrogenophaga* sp. IA3-A

Purification step	Total protein (mg)	Total activity[a] (units)	Specific activity (units/mg)	Yield (%)
Crude extract	395	409	1.04	100
Fast-Flow DEAE Seph.[b]	68	186	2.74	45
Hydroxylapatite	43	57	1.33	14

[a]One unit of enzyme activity defined as 1.0 μmol of oxygen consumed per min.

[b]Fast-Flow DEAE-Sepharose

(Values are mean of duplicate reactions).

Table 5.3. Effect of various aromatic substrates on the activity of partially purified biphenyl dioxygenase from *Hydrogenophaga* sp. IA3-A

Substrate[a]	Specific activity (nmol NADH min^{-1} mg^{-1} protein)
Biphenyl	0.024 ± 0.007
2-monochlorobiphenyl	0.023 ± 0.004
4-monochlorobiphenyl	0.077 ± 0.017
2,4'-dichlorobiphenyl	0.023 ± 0.007
2,4,4'-trichlorobiphenyl	0.007 ± 0.001
2,2',4-trichlorobiphenyl	0.003 ± 0.007
Benzoate	0
4-chlorobenzoate	0.010 ± 0.008
2,3-chlorobenzoate	0.017 ± 0.004
2,6-chlorobenzoate	0.019 ± < 0.001
Benzene	0.027 ± 0.008
1,4-dichlorobenzene	0.006 ± < 0.001
Catechol	0
4-chlorocatechol	0
4-methylcatechol	0
Naphthalene	0
Toluene	0.027 ± 0.003
p-xylene	0.026 ± 0.006
o-xylene	0.033 ± 0.011
m-xylene	0.032 ± 0.001

Composition of reaction mixtures in 2 ml of 50 mM MES buffer (pH 6.0): 6.8 μM $Fe(SO_4)_2(NH_4)_2$; 33.33 μM NADH; 166.66 or 150 μM substrates; and 10 µL of partially purified components A, E, and F.

[a]Concentration or amount of substrate (μM): 166.66, biphenyl; 0.053 % (v/v), benzene, toluene, xylenes; 150, all other substrates. (N = 2 ± SD).

Table 5.4. Effect of FAD concentration on activity of partially purified biphenyl dioxygenase from *Hydrogenophaga* sp. IA3-A

Concentration (μM)	Specific activity[a]
1	0.046 ± 0.003
5	0.034 ± 0
10	0.037 ± < 0.001
20	0.036 ± 0.002
40	0.027 ± 0.005

[a]Specific activity, nmol of NADH oxidized per min per milligram protein (N = 2 ± SD).

Composition of reaction mixtures in 2 ml of 50 mM MES buffer (pH 6.0): 166.66 μM biphenyl; 33.33 μM NADH; 6.8 μM $Fe(SO_4)_2(NH_4)_2$; 1 - 40 μM FAD; and 10 μL of component A, E, and F.

FAD, flavin adenine dinucleotide

Table 5.5. Effect of low temperature and presence of selected aromatic compounds on the activity of partially purified biphenyl dioxygenase from *Hydrogenophaga* sp. IA3-A

Substrates[a]	Specific activity[b]
Biphenyl	0.016 ± 0.008
4-monochlorobiphenyl	0.019 ± 0.007
2,4'-dichlorobiphenyl	0.028 ± 0.005
4-chlorobenzoic acid	0.002 ± 0.001
Benzene	0

[a]Concentration of substrate: biphenyl, 166.66 μM; 2,4'-dichlorobiphenyl, 125 μM; 4-monochlorobiphenyl, 150 μM; 4-chlorobenzoic acid, 150 μM; and benzene, 0.053 % (v/v).

[b]Specific activity, nmol of NADH oxidized per min per milligram protein (N = 2 ± SD). Composition of reaction mixtures in 2 ml of 50 mM MES buffer (pH 6.0) incubated at 7 °C: 0.053 % (v/v) or 125 – 166.66 μM substrate; 33.33 μM NADH; 6.8 μM $Fe(SO_4)_2(NH_4)_2$; 1.0 μM FAD; and 10 µL of partially purified components A, E, and F.

Note that FAD was included in the reaction mixtures

Table 5.6. Thermostability of the partially purified biphenyl dioxygenase from *Hydrogenophaga* sp. IA3-A

Temperature (°C)[a]	Specific activity[b]	%[c]
0	0.057 ± 0.018	100
10	0.047 ± 0.003	82
20	0.040 ± 0.005	70
30	0.041 ± 0.006	72
35	0.032 ± 0.005	56
40	0.027 ± 0.008	47
53	0.024 ± 0.012	42
63	0.013 ± 0.003	27
65	0	0

[a]The partially purified BPDO components were suspended in 50 mM MES buffer (pH 6.0) and incubated for two minutes in a water-bath maintained at the desired temperature prior to initiation of reaction at 30 °C

[b]Specific activity, nmol of NADH oxidized per min per milligram protein (N = 2 ± SD).

[c]Activity expressed as a percentage of maximum specific activity

Composition of reaction mixtures in 2 ml of 50 mM MES buffer (pH 6.0): 166.66 μM biphenyl; 33.33 μM NADH; 6.8 μM $Fe(SO_4)_2(NH_4)_2$; 1 μM FAD; 7 μL of partially purified component A and F, and 5 μL of partially purified component E.

Table 5.7. Effect of solvent, chelating agent, and metal ions on the activity of the partially purified biphenyl dioxygenase from *Hydrogenophaga* sp. IA3-A

Solvent/EDTA/Metal ion[a]	Specific activity[b]	%[c]
Triton X-100	0.025 ± 0.004	54
Tween 80	0.029 ± 0.006	63
Acetone	0.018 ± 0.001	39
EDTA	0.024 ± 0.003	52
Zinc chloride	0.031 ± 0.001	67
Cobalt chloride	0.025 ± 0.006	54
Mercury chloride	0.001 ± 0.002	2
Nickel sulphate	0.016 ± 0.002	35
Sodium molybdate	0.028 ± 0.009	61
Ferric chloride	0.030 ± 0.005	65
Copper sulphate	0	0
Manganese chloride	0.031 ± 0.006	67
Magnesium chloride	0.035 ± 0.002	76
Biphenyl only	0.046 ± 0.003	100

[a]Amount or Concentration of test agent: 0.1 % (v/v) Triton X-100, Tween 80, or acetone; 250 μM EDTA (ethylenediaminetetraacetic acid); 250 μM of metal ions.

[b]Specific activity, nmol of NADH oxidized per min per milligram protein (N = 2 ± SD).

[c]Activity expressed as a percentage of specific activity in the presence of biphenyl only.

Composition of reaction mixtures in 2 ml of 50 mM MES buffer (pH 6.0): 166.66 μM biphenyl; 33.33 μM NADH; 6.8 μM $Fe(SO_4)_2(NH_4)_2$; 1 μM FAD; and 10 μL of partially purified component A, E, and F.

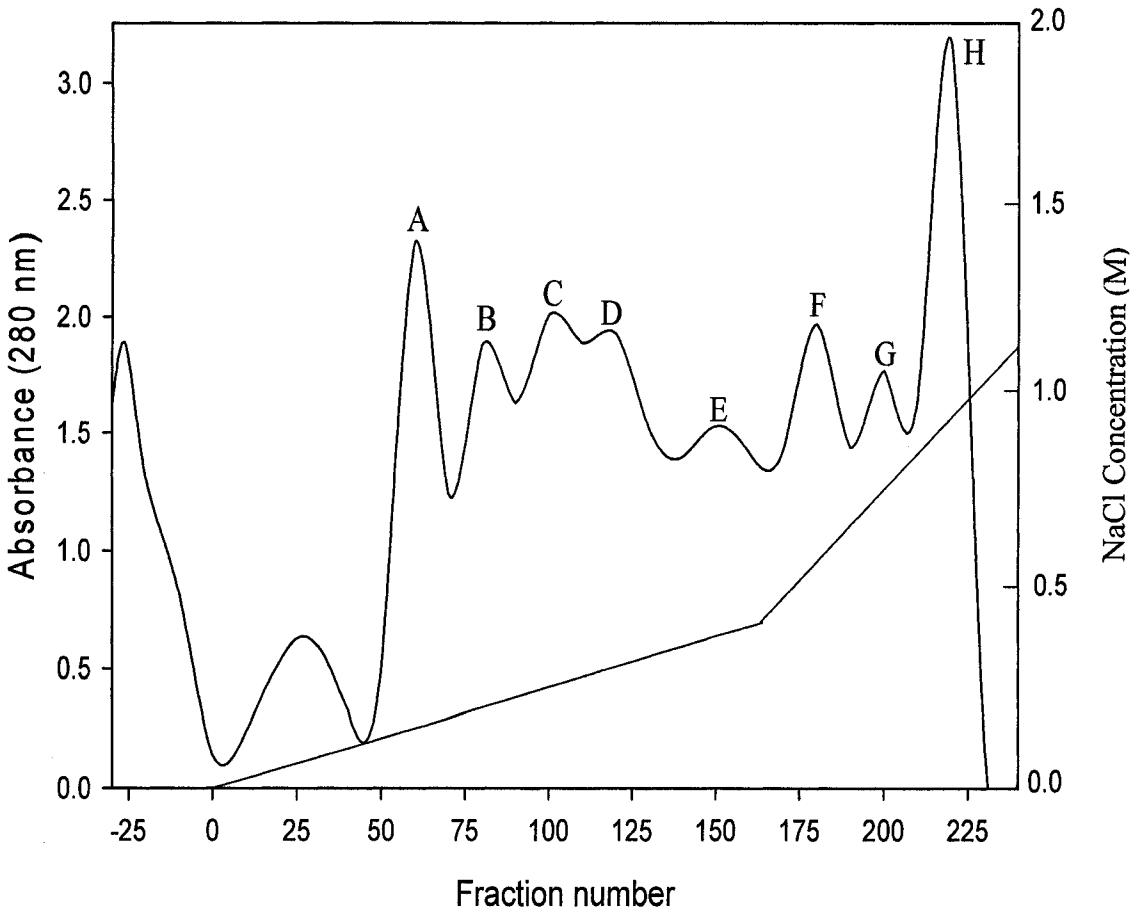


Fig. 5.1. Ion-exchange chromatography of biphenyl-induced cell extract of *Hydrogenophaga* sp. IA3-A on a Fast-Flow DEAE-Sepharose column using a linear salt gradient from the starting buffer to 0.4 M NaCl, followed by a second gradient of 0.4 to 2.0 M NaCl. Fractions collected: components A, 48 – 71; B, 72 – 88; C, 89 – 110; D, 111 – 125; E, 126 – 171; F, 172 – 193; G, 194 – 208; H, 209 – 230.

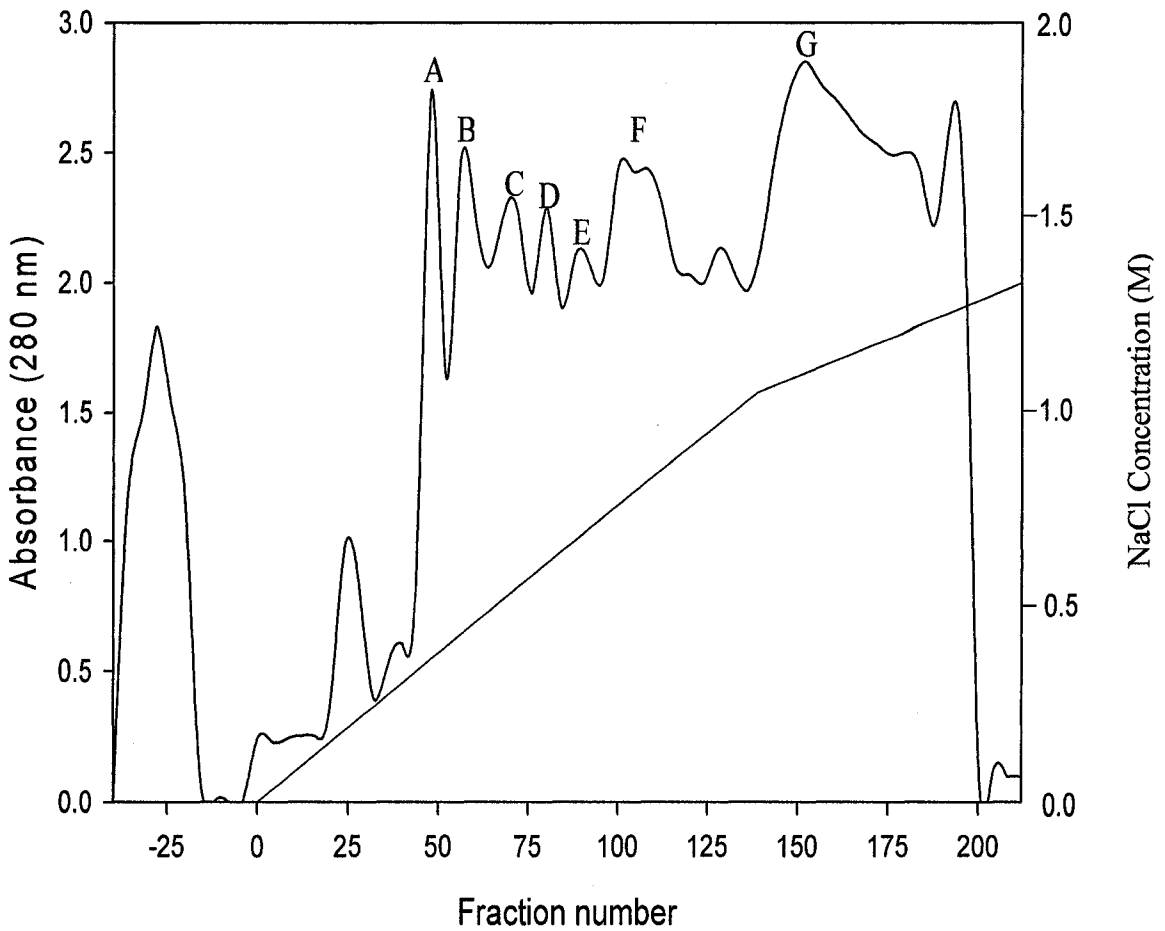


Fig. 5.2. Ion-exchange chromatography of biphenyl-induced cell extract of *Hydrogenophaga* sp. IA3-A on a Fast-Flow DEAE-Sepharose column using a linear salt gradient from the starting buffer to 0.3 M NaCl, followed by a second gradient of 0.3 to 2.0 M NaCl. Fractions collected: components A, 42 – 57; E, 76 – 94; F, 94 – 122;

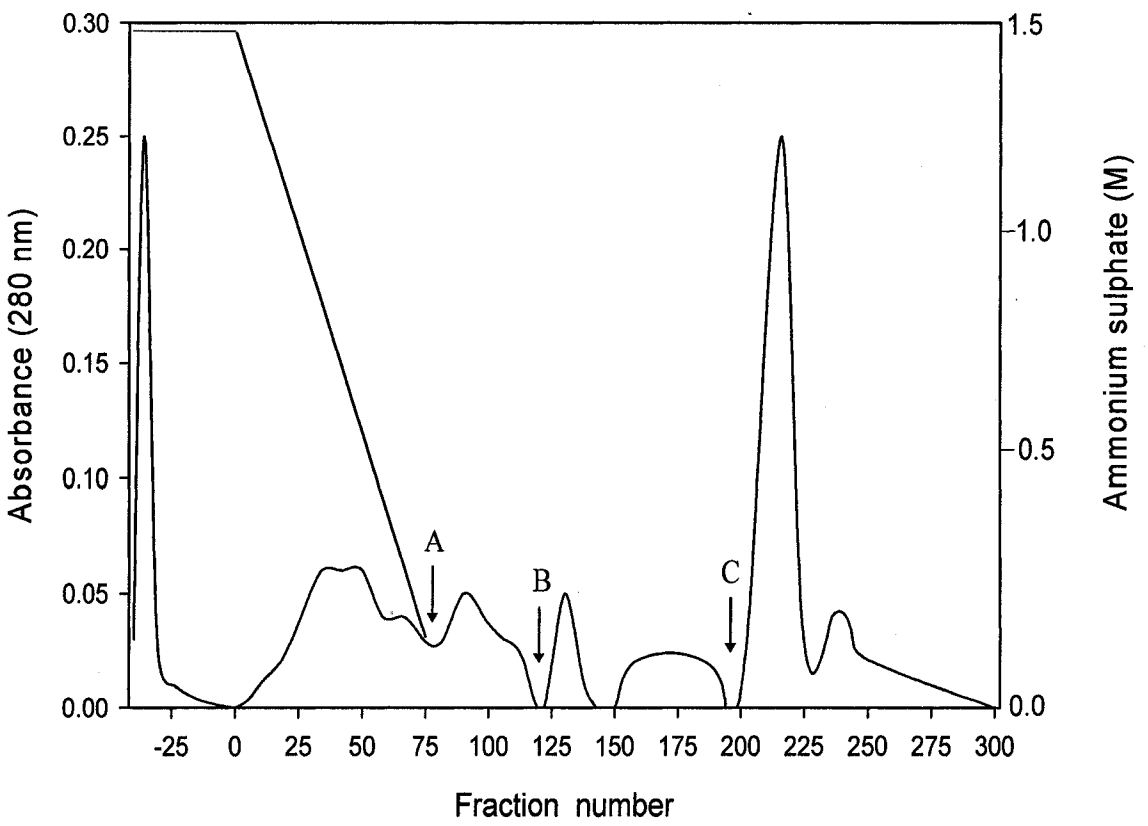


Fig. 5.3. Hydrophobic interaction chromatography of oxygenase component (E) from DEAE-Sepharose column on a Octyl-Sepharose CL-4B column using a decreasing salt gradient from 1.5 to 0.0 M NH_4SO_4 dissolved in 25 mM MES buffer (pH 6.0) containing 10 % glycerol; and equilibration buffer (A), equilibration buffer plus 50 % (v/v) ethylene glycol (B), and equilibration buffer plus 30 % (v/v) isopropanol (C). Note that eluates were lost during dialysis.

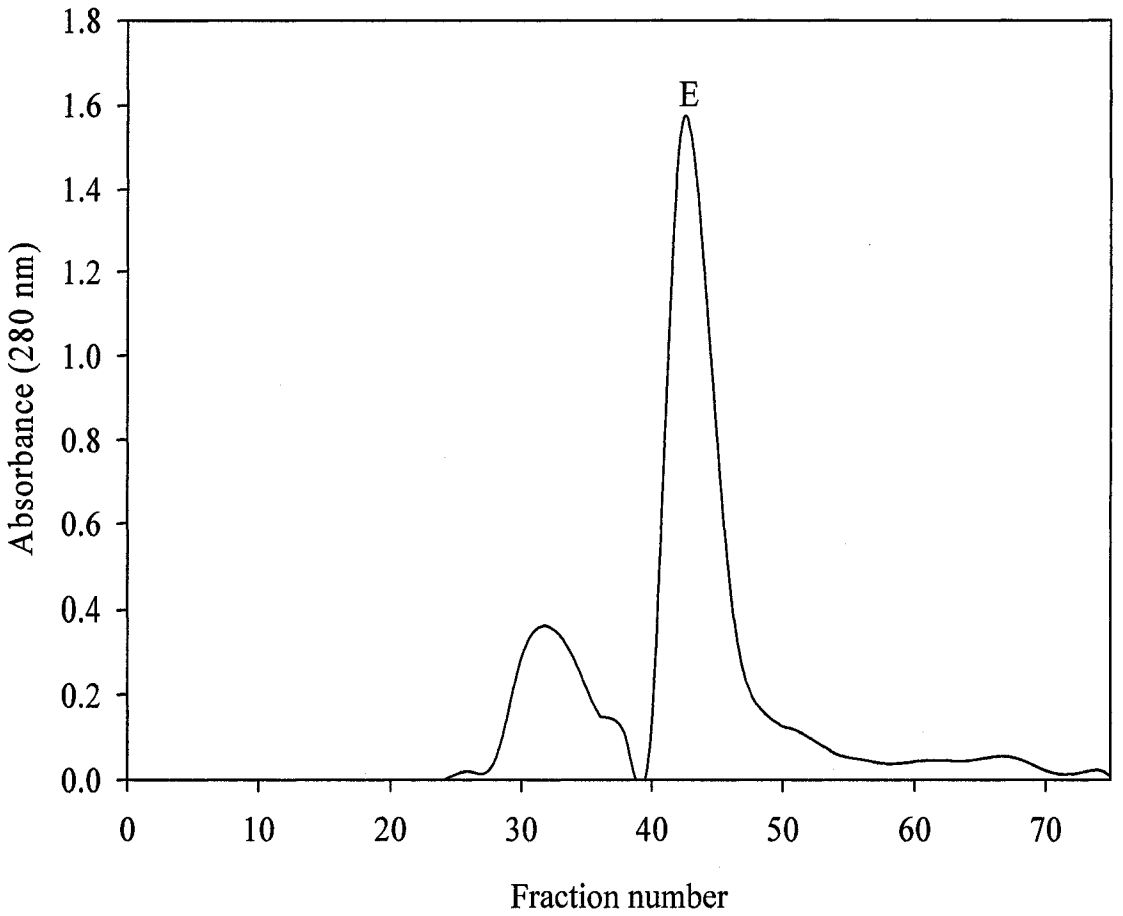


Fig. 5.4. Elution profile of the partially purified oxygenase component (E) from a hydroxylapatite column using 100 mM potassium phosphate (pH 7.0), and 250 mM potassium phosphate (pH 7.0). The oxygenase component was previously eluted from a Fast-Flow DEAE-Sepharose column.

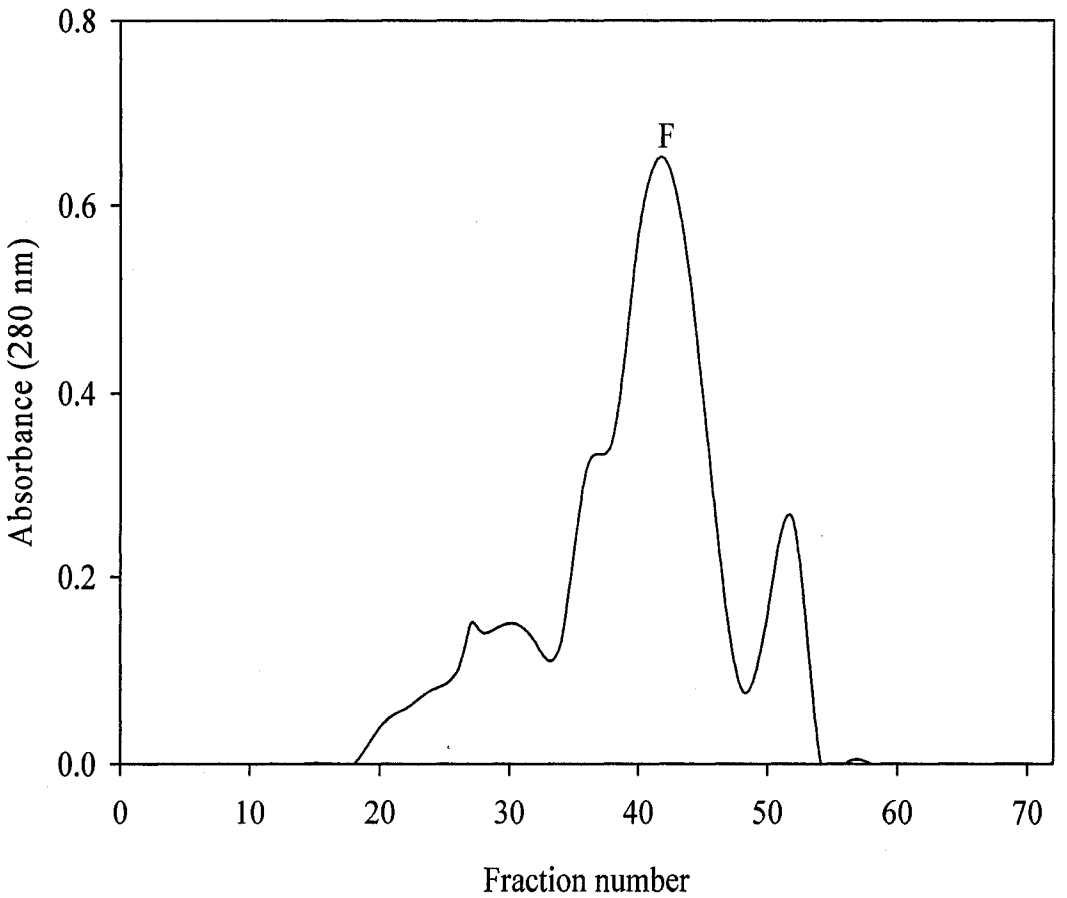


Fig. 5.5. Elution profile of the partially purified ferredoxin component (F) from a hydroxylapatite column using 100 mM potassium phosphate (pH 7.0) and 250 mM potassium phosphate (pH 7.0). The ferredoxin component was previously eluted from a Fast-Flow DEAE-Sepharose column.

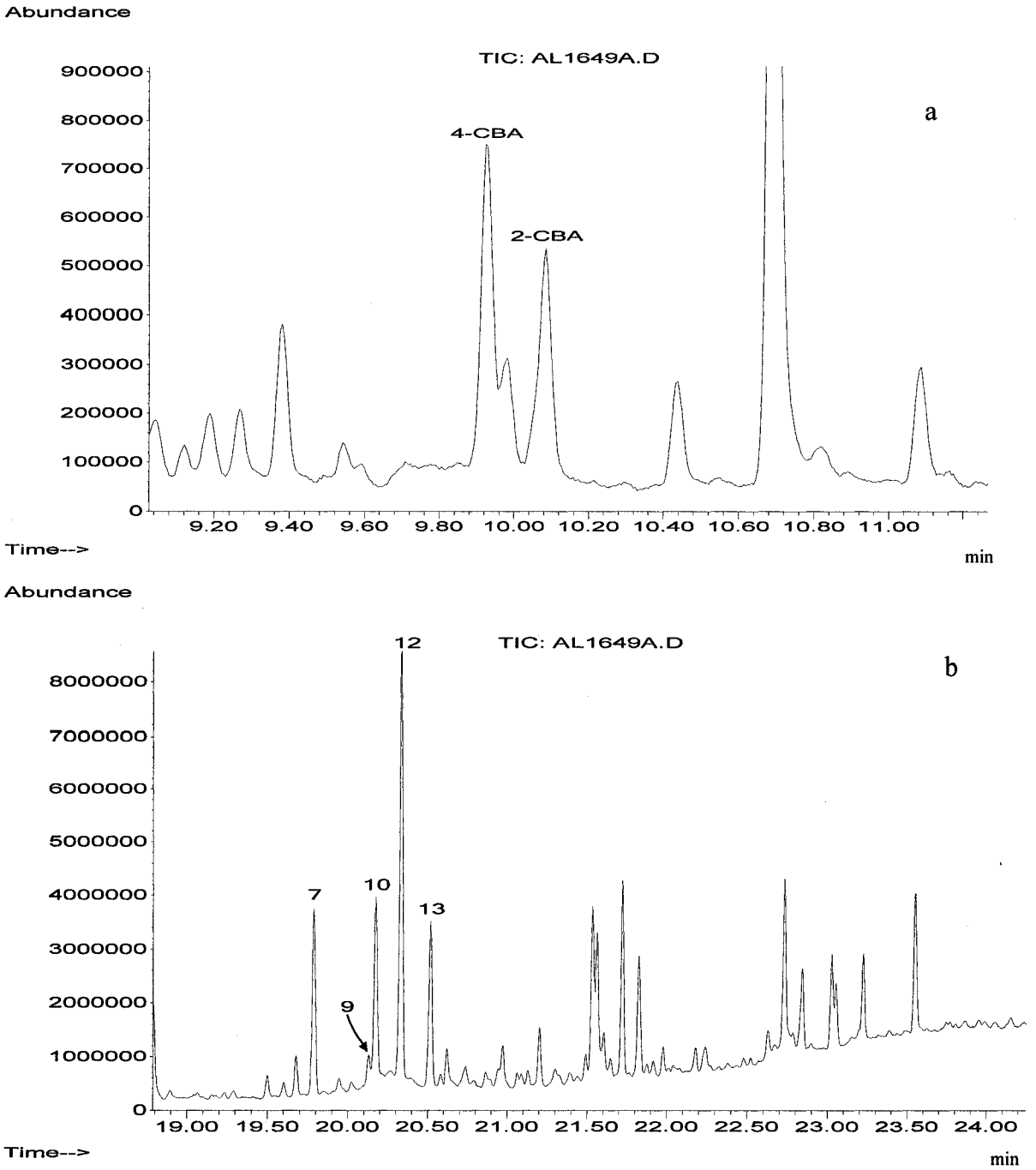


Fig. 5.6. Sections of the TIC of extracts of cultures incubated with 2,4'-DiCB at 55 °C for 48 h showing 2- and 4-CBA (a), and hydroxylated intermediates (b) (peaks nos. 7, 9, 10, 12, and 13). Proposed structures of annotated peaks: monochloro-dihydroxybiphenyls, peak nos. 7, 9, 10 and 12; dichloro-dihydrodiol, peak no. 13. Peak nos. are the same as those shown in the footnote of Table 4.2.

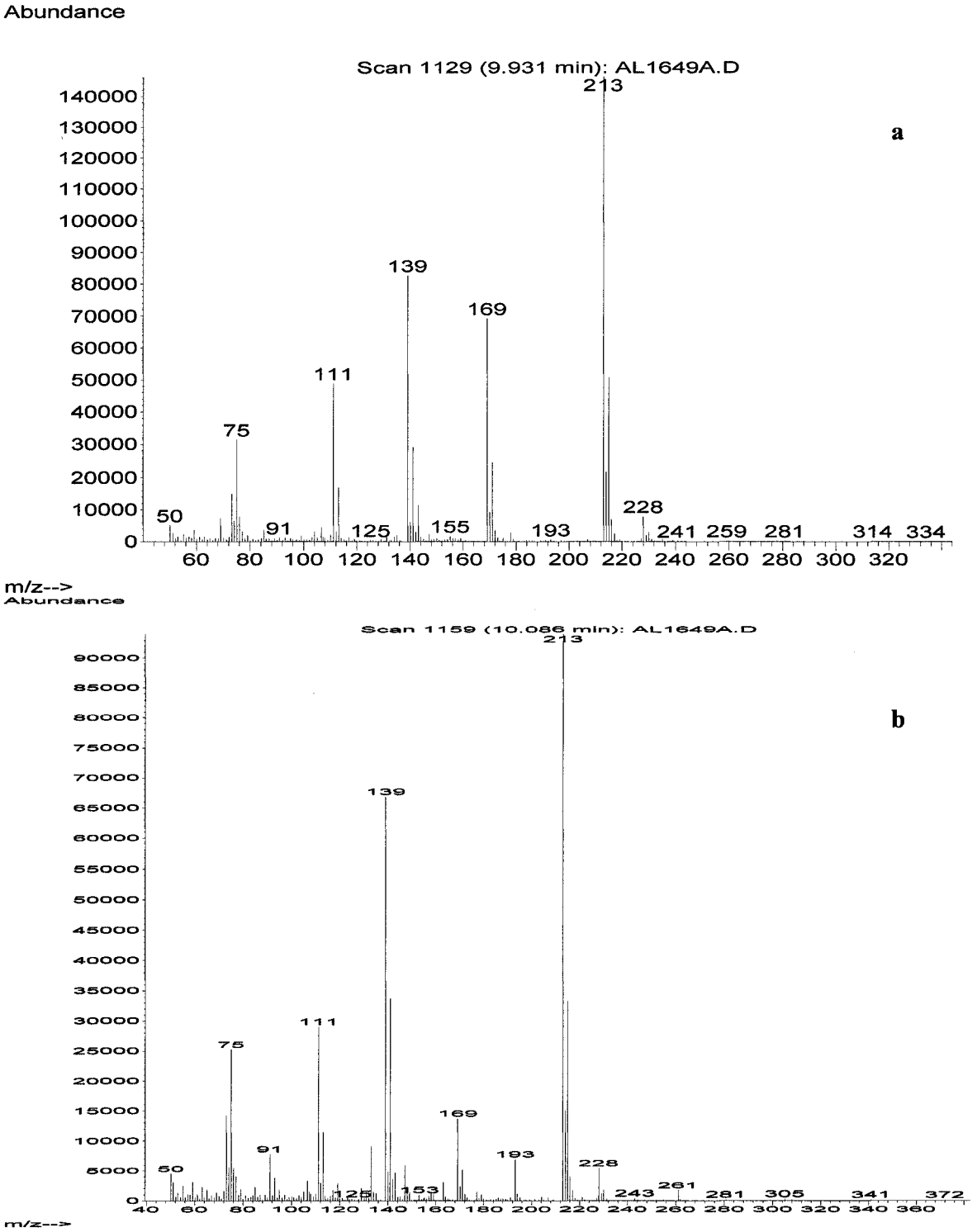


Fig. 5.7. Mass spectra of 4-CBA (a) and 2-CBA (b) formed from 2,4'-DiCB *Hydrogenophaga* sp. IA3-A incubated at 55 °C for 48 h.

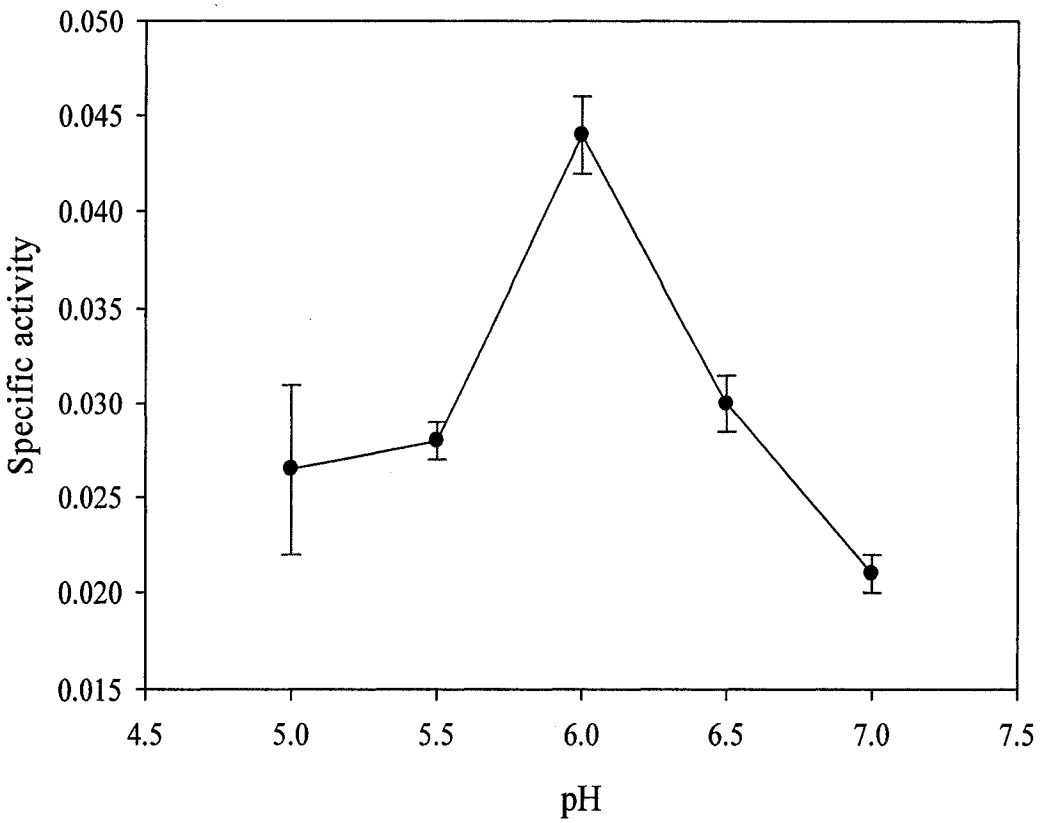


Fig. 5.8. Effect of pH on the specific activity of the partially purified biphenyl dioxygenase from *Hydrogenophaga* sp . IA3-A. Data points are mean values from duplicate reactions. Error bars indicates standard deviation.

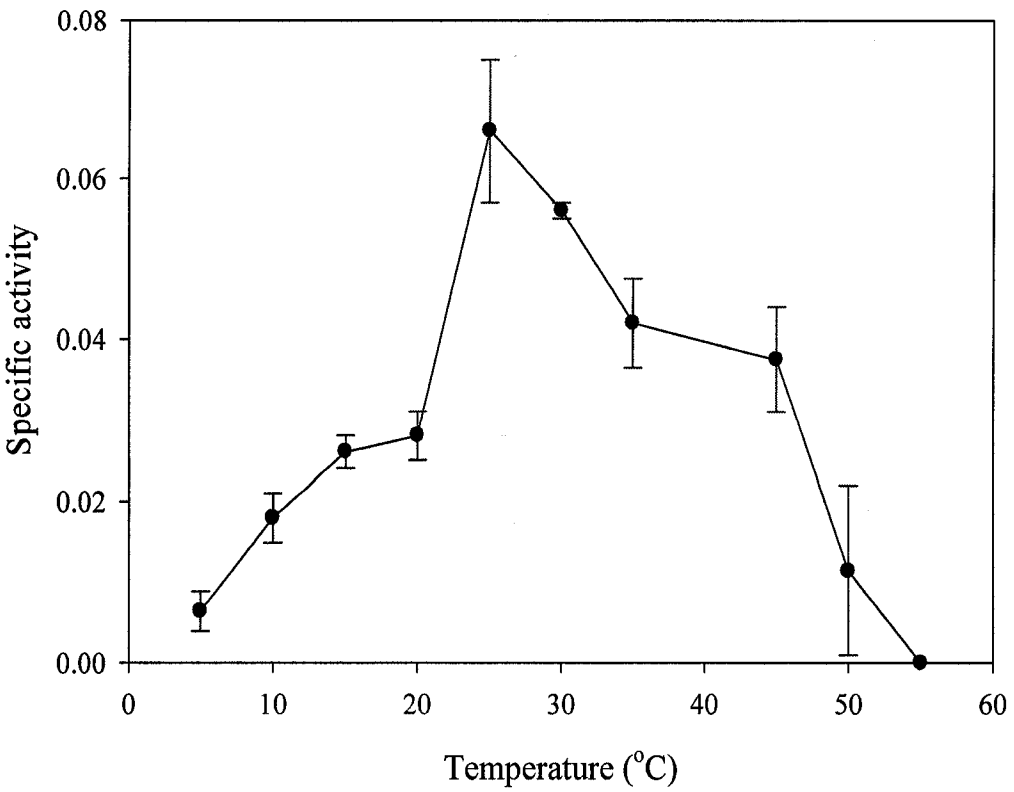


Fig. 5.9. Effect of temperature on the specific activity of the partially purified biphenyl dioxygenase from *Hydrogenophaga* sp . IA3-A. Data points are mean values from duplicate reactions. Error bars indicates standard deviation.

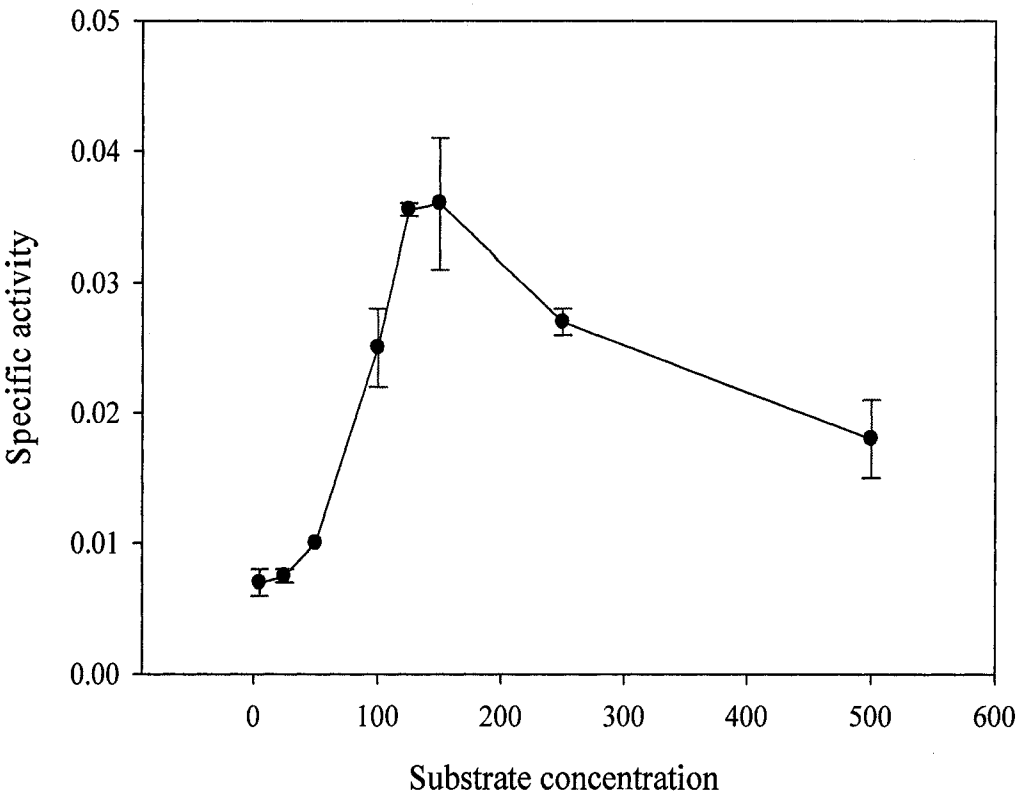


Fig. 5.10. Effect of substrate concentration on the specific activity of the partially purified biphenyl dioxygenase from *Hydrogenophaga* sp . IA3-A. Data points are mean values from duplicate reactions. Error bars indicates standard deviation.

5.4. Discussion

The BPDO from several mesophilic strains have been purified and characterized (Imbeault *et al.*, 2000; Hurtubise *et al.*, 1995; Haddock *et al.*, 1993) and transformation of chlorinated biphenyls by the BPDO from strain LB400 have also been studied. Although, induction of *bphA*, the gene encoding the BPDO of psychrotolerant *Pseudomonas* strain Cam-1 has been studied, but there are no reports describing purified or partially purified BPDOs from cold-adapted PCB-degrading bacterial strains. Like other aromatic-ring hydroxylating dioxygenases, the multicomponent system of BPDO has been described in previous studies (Haddock *et al.*, 1993). Studies on the individual components have also been carried out (Broadus and Haddock, 1998; Haddock and Gibson, 1995; Hurtubise *et al.*, 1995). It has also been shown in the present studies that the partially purified BPDO of *Hydrogenophaga* sp. IA3-A required fraction A, E, and F (Table 5.1) for maximum activity.

As noted in previous studies (Haddock and Gibson, 1995; Haddock *et al.*, 1993), fraction A regarded as the reductase was yellowish, while fractions E and F regarded as the oxygenase and ferredoxin components, respectively, were brownish. In addition, component E was confirmed to display characteristic absorbance at 323 and 455 nm during separation of protein fractions using DEAE-Sepharose and hydroxylapatite. A measurement of absorbance of protein fractions at 277 nm was also used to separate components E and F.

A comparison of the activity of BPDO in the presence of biphenyl only (Table 5.3) to the activity of BPDO in the presence of biphenyl and FAD (flavin adenine dinucleotide) (Table 5.4) showed that lower concentration of the cofactor stimulated

BPDO activity. FAD was shown previously to stimulate BPDO activity in cell extract from strain LB400 (Haddock *et al.*, 1993). The effect of FAD on the activity of BPDO could be related to its ability to stimulate electron transfer from the reductase to the oxygenase component, as shown for the reductase and oxygenase components of toluene dioxygenase from *Pseudomonas putida* (Subramanian *et al.*, 1981). In contrast to the observation here, Hurtubise et al. (1995) showed that increased concentration of FAD increased the oxidation of NADH, but it resulted in decreased formation of dihydrodiol, the product of oxidation of biphenyl by BPDO. The ability of FAD to stimulate electron transfer is known to depend on the concentration of reductase and oxygenase (Subramanian *et al.*, 1981). Likewise, Hurtubise *et al.* (1995) suggested the possibility that FAD could act as an electron sink or substrate for the oxygenase.

The partially purified BPDO of strain IA3-A oxidized NADH in presence of some aromatic substrates at a similar rate as in the presence of biphenyl, 2-MonoCB, and 2,4'-DiCB (Table 5.3). The enzyme also oxidized NADH in the presence of chlorinated benzoates instead of benzoate. In contrast, the enzyme preferred benzene instead of chlorinated benzene. Oxidation of NADH by BPDO from *Comamonas testosteroni* B-356 in the presence of benzene has been observed before (Hurtubise *et al.*, 1995), but the same enzyme failed to oxidize toluene and naphthalene. Poor oxidation of NADH in the presence of 2,4,4'- and 2,2',4-TriCB indicates that activity of BPDO of strain IA3-A in the presence of chlorinated biphenyls is determined by the number and pattern of chlorination of the compounds. Previously characterized BPDOs have not been shown to be active against aromatic substrates at low temperature. Results in Table 5.5 show that

BPDO of *Hydrogenophaga* sp. IA3-A was active against some aromatic compounds at low temperature. This is understandable since strain IA3-A is a cold-adapted strain.

The BPDO of strain IA3-A was relatively thermo-stable and cells formed metabolic products from 2,4'-diCB when incubated at 55 ^{o}C for 48 h (Fig. 5.6, Fig. 5.7). It is likely that biphenyl-degrading enzymes in cold-adapted PCB-degrading strains are thermo-stable; since PCBs were degraded at 50 ^{o}C by some psychrotolerant strains (Master and Mohn, 1998). The activity of BPDO was not severely affected when the enzyme was incubated at 60 ^{o}C for 2 min, but it was observed that reaction mixtures became cloudy after incubation at higher temperature. This could be due to denaturation of the protein in the mixtures.

The inhibition of the activity of BPDO by Tween 80 and Triton X-100 is similar to the results obtained with other dioxygenase systems (Hurtubise et al., 1995; Subramanian *et al.*, 1985). Unlike in those studies, low amount of acetone inhibited activity here. All the metal ions tested also inhibited BPDO activity, but the effect of Hg^{2+} and Cu^{2+} was more severe. The conversion of biphenyl into dihydrodiol products by the BPDO from a mesophilic strain was strongly inhibited by these ions (Hurtubise *et al.*, 1995). The apparent K_m toward biphenyl by the BPDO from strain IA3-A is within the range of the values reported for some chimeric BPDOs (Hurtubise *et al.*, 1998). The curve obtained by examination of enzyme kinetics and substrate concentration was non-hyperbolic (Fig. 5.10), suggesting that inhibition of BPDO by excess amount of biphenyl occurred.

Chapter 6

General Discussion and Conclusion

Microbial degradation of PCBs has received widespread attention as a result of the recalcitrant nature of PCBs, but of most these studies were directed toward mesophilic strains. The presence of PCBs in cold climates and the remoteness of contaminated sites dictate that decontamination of PCBs at these sites would only be successful by using cold-adapted strains. Before now, only two studies investigated the removal of PCBs by cold-adapted strains at low temperature using strains that are closely related to the genus *Pseudomonas* (Master and Mohn, 1998; Mohn *et al.*, 1997). Here, a new cold-adapted PCB-degrading strain was isolated from PCB-contaminated soils in a site with temperate climates. Strain IA3-A is the first cold-adapted strain belonging in the genus *Hydrogenophaga* found to cometabolize or grow on aromatic compounds. This study is also the first to report the degradation of chlorinated aromatic compounds by a strain belonging in the genus *Hydrogenophaga.*

Like most PCB-degrading bacteria, *Hydrogenophaga* sp. IA3-A could only cometabolize lightly chlorinated PCBs. Double *ortho-* substituted congeners like 2,2'- and 2,6- were resistant to degradation. Surprisingly, strain IA3-A did not degrade 4,4'-diCB efficiently. Most strains that degraded 2,2'- or 2,6-diCB poorly could degrade 4,4'-diCB efficiently (Bedard *et al.*, 1987b; Bedard *et al.*, 1986). In addition removal of 4,4'-diCB at low temperature appear to depend on the composition of PCB mixtures since the congener was moderately degraded in Aroclor 1221 at low temperature but the same compound was not degraded in Aroclor 1232. Addition of a single *ortho-* chlorine to 4,4'-diCB to give 2,4,4'-triCB enhanced the initial attack of PCB by strain IA3-A, but this effect was more

pronounced in Aroclor 1232. Results also show that addition of a single *para*- chlorine to 2,2'-diCB to give 2,2',4-triCB slightly enhanced the initial attack of the PCB by strain IA3-A at moderate temperature only. The inability of the bacterium to degrade highly chlorinated congeners or congeners like 2,2'-diCB and 2,5,2'-triCB indicates that it attacked PCBs using biphenyl-2,3-dioxygenase. The enzymes responsible for degradation of (chloro)biphenyl were induced to higher levels in cells of *Hydrogenophaga* sp. IA3-A grown on biphenyl. The enzymes were constitutively expressed at low levels when cells were grown on other carbon sources like succinate, and most likely acetate and pyruvate.

PCB-degrading enzymes exhibit different level of sensitivity to their respective substrates (Dai *et al.*, 2002; Seah *et al.*, 2000). Therefore, the removal of individual congeners does not suggest complete transformation of the compounds. In addition, screening of metabolic products can reveal the regiospecificity of attack of PCBs (Seegar *et al.*, 1995). Formation of metabolic products during low temperature degradation of PCBs has not been demonstrated before now. Analysis of the nature of metabolic products that were formed from mixtures of PCBs or the individual congeners indicates that *Hydrogenophaga* sp. IA3-A preferentially attacked PCBs at phenyl rings bearing *para*- chlorines. There are two lines of evidence in support of this conclusion. These are the relatively higher levels of *ortho*-substituted HOPDAs that are formed from PCBs mixtures, and the high levels of *ortho*-substituted HOPDAs that are formed from 2,4'-diCB and 2,4,4'-triCB. But the low levels of *para*-substituted HOPDAs that are formed from PCB mixtures, including the production of 4-CBA from 2,4'-diCB or 2,4,4'-triCB indicates that the bacterium could also oxidize chlorinated rings bearing an *ortho*- chlorine.

Some HOPDAs are inhibitory to the enzyme responsible for their conversion. A HOPDA compound bearing chlorine on the dienoate moiety competitively inhibited HOPDA-hydrolase (Seah *et al.*, 2000; Seah *et al.*, 2001). HOPDAs that are formed from 2,4'-diCB and 2,4,4'-triCB bears chlorine on their dienoate moiety. Chlorinated HOPDAs are also precursors of highly toxic chloroacetophenones (Baxter and Sutherland, 1984), which could also generate toxic chlorophenols (Havel and Reineke, 1993). In this light, studies directed toward understanding the conditions that prevents accumulation of HOPDAs from PCBs is warranted. The formation of 2- and 4-CBA from 2,4'-diCB indicates that *Hydrogenophaga* sp. IA3-A could oxidize both rings of the compound, though to different extents. This observation, combined with the formation of isomeric hydroxylated products from 2,4'-diCB (and even 2,4,4'-triCB) suggest that multiple routes were used for the transformation of the congener. Results here suggest that temperature regulates the pattern of transformation of the compound. The removal of PCBs at various temperatures by cold-adapted strains and mesophilic strain LB 400 has been reported before (Master and Mohn, 1998), but there is yet no report on the effect of temperature on the pattern of transformation of PCBs. The effect of higher temperature (e.g. 45 °C) on the pattern of transformation of 2,4'-diCB could be related to the activation or inhibition of enzymes of the pathway. It is likely that the - subsequent - metabolic flux relieved the bottlenecks along the pathway, thus resulting in efficient transformation of the degraded 2,4'-diCB. Evidence in support of this assumption is that at 45 °C, the level of HOPDA was very low and that 87 % of the 2,4'-diCB that was degraded was recovered as CBAs (Table 4.1). The remaining 13 % of the degraded compound could be accounted for by the low level of HOPDA and the hydroxylated intermediates that were detected in supernatants at this temperature (see Fig.

4.4b).

It is astonishing that more than twenty chlorinated intermediates were formed from 2,4'-diCB. Some of these products could have been formed as a result of non-specific attack of 2,4'-diCB by strain IA3-A. Formation of multiple isomeric hydroxylated products is peculiar to enzyme systems that exhibit non-specific attack against aromatic compounds (Lindner *et al.*, 2000). The possibility that some of the products detected here were generated by attack of 2,4'-diCB by non-specific monooxygenase systems in strain IA3-A cannot be disproved at this point. A confirmation of the purity of stock solution of 2,4'-diCB ruled out the possibility that some of these products (e.g. monochloro-dihydroxybiphenyl) were generated from impurities in the solution. It is important to note that similar (dichloro) dihydroxylated products were produced from 2,4,4'-triCB. A plausible explanation is that some of the products were produced as artifacts during derivatization. Additional studies using purified biphenyl-degrading enzymes from strain IA3-A are required. Cloning of biphenyl catabolic genes from strain IA3-A should permit the characterization of the routes of formation of these intermediates.

A more detailed and exhaustive characterization of partially purified biphenyl-2,3-dioxygenase (BPDO) from *Hydrogenophaga* sp. IA3-A, as well as investigation of the transformation of 2,4'-diCB by the enzyme at different temperature was not possible. The enzyme was active at low temperature and it was active against different classes of aromatic compounds. Surprisingly, the enzyme was active against chlorobenzoates (CBAs) (Table 5.3), but not against benzoate. The BPDO of strain IA3-A was not expected to be active against CBAs, taking into consideration that biphenyl-grown cells failed to oxidize the compounds. The transport of CBAs into cells probably requires a factor that was not present

in biphenyl-grown cells. At moderate temperature, the enzyme was more active against 4-monoCB than biphenyl and other chlorobiphenyls. But its activity against 4-monoCB was decreased at low temperature. It is interesting to note that the enzyme retained significant activity against 2,4'-diCB at low temperature. The adopted conformation of BPDO at low temperature and the presence of two chlorine atoms might have enhanced the alignment of 2,4'-diCB in the active site of the enzyme.

The present study isolated a PCB-degrading strain belonging to a genus that is poorly characterized for the ability of its members to degrade aromatic compounds. This study showed that the strain degraded PCBs at low temperature. The effects of low temperature on the pattern of attack of PCBs were reported. For the first time, direct evidence was provided in support of products formation from PCBs at low temperature. This study also showed that excess accumulation of problematic HOPDA(s) from a model PCB compound can be avoided under specific conditions. Attempts were also made to characterize the cold-adapted BPDO from strain IA3-A, which is critical to biological decontamination of PCBs in cold climates. The results here raise the question as to whether accumulation of HOPDAs from some PCBs by other PCB-degrading strains is avoidable under specific conditions. Therefore, studies are required to investigate the transformation of PCBs by these strains under different (temperature) conditions. Further studies investigating the transformation of PCBs using strain IA3-A should include analytical techniques like NMR (Nuclear Magnetic Resonance) and HRMS (High Resolution Mass Spectrometry). Complete elucidation of the pathways leading to the formation of the metabolic products detected here would require purification of (chloro)biphenyl degrading enzymes of *Hydrogenophaga* sp. IA3-A, as well as the use of authentic standards of the likely products of the pathways.

References

Abbott, T.P., and Wicklow, D.T. (1984). Degradation of lignin by *Cyathus* species. Appl. Environ. Microbiol. **47**: 585-587.

Abramowicz, D.A. (1990). Aerobic and anaerobic biodegradation of PCBs: A review. Crit. Rev. Biotechnol. **10**: 241-251.

Adriaens, P., Kohler, H.P.E., Kohler-Staub, D., and Focht, D.D. (1989). Bacterial dehalogenation of chlorobenzoates and coculture biodegradation of 4,4'-dichlorobiphenyl. Appl. Environ. Microbiol. **55**: 887-892.

Afferden, M., Schacht, S., Klein, J., and Truper, H.G. (1990). Degradation of dibenzothiopene by *Brevibacterium* sp. DO. Arch. Microbiol. **153**: 324-328.

Ahmed, M., and Focht, D.D. (1973). Degradation of polychlorinated biphenyls by two species of *Achromobacter*. Can. J. Microbiol., **19**: 47-52.

Ahmad, D., Sylvestre, M., and Sondossi, M. (1991). Subcloning of bph genes from *Pseudomonas testosterone* B-356 in *Pseudomonas putida* and *Escherichia coli*: Evidence for dehalogenation during initial attack on chlorobiphenyls. Appl. Environ. Microbiol. **57**: 2880-2887.

Aislabie, J., Foght, J., and Saul, D. (2000). Aromatic hydrocarbon-degrading bacteria from soil near Scott base, Antarctica. Polar Biol. **23**: 183-188.

Albro, P.W., Corbett, J.T., and Schroeder, J.L. (1981). Quantitative characterization of polychlorinanated biphenyl mixtures (Aroclors 1248, 1254 and 1260) by gas chromatography using capillary columns. J. Chromatogr. **136**:147-153.

Alder, A.C., Haggblom, M.M., Oppenheimer, S.R., and Young, L.Y. (1993). Reductive dechlorination of polychlorinated biphenyls in anaerobic sediments. Environ. Sci.

Technol. **27**: 530-538.

Arensdorf, J.J., and Focht, D.D. (1994). Formation of chlorocatechol *meta* cleavage products by a pseudomonad during metabolism of monochlorobiphenyls. Appl. Environ. Microbiol. **60**: 2884-2889.

Assinder, S.J., and Williams, P.A. (1990). The *tol* plasmids: determinants of catabolism of toluene and xylenes. Adv. Microbial. Physiol. **31**: 1-69.

Atlas, R.M. (1981). Microbial degradation of petroleum hydrocarbons: an environmental perspective. Microbiol. Rev. **45**: 180-209.

Bamford, H.A., Poster, D.L., and Baker, J.E. (2000). Henry's law constant of polychlorinated biphenyl congeners and their variation with temperature. Environ. Sci. Technol. **45**: 1069-1074.

Baraniecki, C.A., Aislabie, J., and Foght, J.M. (2002). Characterization of *Sphingomonas* sp. Ant 17, an aromatic hydrocarbon-degrading bacterium isolated from Antarctic soil. Microb. Ecol. **43**: 44-54.

Barriault, D., Plante, M.M., and Sylvestre, M. (2002). Family shuffling of a targeted *bphA* region to engineer biphenyl dioxygenase. J. Bacteriol. **184**: 3794-3800.

Barton, M.R., and Crawford, R.L. (1988). Novel biotransformation of 4-chlorobiphenyl by a *Pseudomonas* sp. Appl. Environ. Microbiol. **54**: 594-595.

Baxter, R.M., and Sutherland, D.A. (1984). Biochemical and photochemical processes in the degradation of chlorinated biphenyls. Environ. Sci. Technol. **18**:608-610.

Bayly, R.C., and Barbour, M.G. (1984). The degradation of aromatic compounds by the meta and gentisate pathways: biochemistry and regulation. In *microbial degradation of organic compounds.* ed. Gibson D.T. Chapter 8, pp. 253-294. Marcel Dekker Inc.

New York, Basel.

Bedard, D.L., Untermann, R., Bopp, L.H., Brennan, M.J., Haberl, M.L., and Johnson, C. (1986). Rapid assay for screening and characterizing microorganisms for the ability to degrade polychlorinated biphenyls. Appl. Environ. Microbiol. **51**: 761-768.

Bedard, D.L., Haberl, M.L., May, R.J., and Brennan, M.J. (1987a). Evidence for novel mechanisms of polychlorinated biphenyl metabolism in *Alcaligenes eutrophus* H850. Appl. Environ. Microbiol. **53**: 1103-1112.

Bedard, D.L., Wagner, R.E., Brennan, M.J., Haberl, M.L., and Brown, Jr. J.F. (1987b). Extensive degradation of Aroclors and environmentally transformed polychlorinated biphenyls by *Alcaligenes eutrophus* H850. Appl. Environ. Microbiol. **53**: 1094-1102.

Bedard, D.L., and Haberl, M.L. (1990). Influence of chlorine substitution pattern on the degradation of polychlorinated biphenyls by eight bacterial strains. Microb. Ecol. **20**: 87-102.

Bedard, D.L., and Quensen J.F. (1995). Microbial reductive dechlorination of polychlorinated biphenyls. In *Microbial Transformation and Degradation of Toxic Organic Chemicals*, ed. L.Y. Young and C.E. Cerniglia, pp. 127-216. Wiley-Liss, Inc., New York.

Beddesem, M., Swoboda-Colberg, N., and Colberg, P. (1997). Naphthalene mineralization coupled to sulphate-reduction in aquifer-derived enrichments. FEMS Microbiol. Lett. **152**: 213-218.

Bej, A.K., Saul, D., and Aislabie, J. (2000). Cold-tolerant alkane-degrading *Rhodococcus* species from Antarctica. Polar Biol. **23**: 100-105.

Bergey, D. H. (1994). Bergey's Manual of Determinative Bacteriology, eds. J. G. Holt,

N. R. Krieg, P. H. A. Sneath, J. T. Staley, and S. T. Williams. 9th edn. Williams and Wilkins, Baltimore.

Berry, D.F., Francis, A.J., and Bollag, J.M. (1987). Microbial metabolism of homocyclic and heterocyclic aromatic compounds under anaerobic conditions. Microbiol. Rev. **51**: 43-59.

Bevinakatti, B.G., and Ninnekar, H.Z. (1993). Biodegradation of 4-chlorobiphenyl by *Micrococcus* species. World J. Microbiol. Biotechnol. **9**: 607-608.

Billingsley, K.A., Backus, S.M., Juneson, C., and Ward, O.P. (1997). Comparison of the degradation patterns of polychlorinated biphenyl congeners in Aroclors by *Pseudomonas* strain LB400 after growth on various carbon sources. Can. J. Microbiol. **43**: 1172-1179.

Billingsley, K.A., Backus, S.M., and Ward, O.P. (1999). Production of metabolites from chlorobiphenyls by resting cells of *Pseudomonas* strain LB400 after growth on different carbon sources. Can. J. Microbiol. **45**: 178-184.

Blumel, S., Contzen, M., Lutz, M., Stolz, A., and Knackmuss, H.-J. (1998). Isolation of a bacterial strain with the ability to utilize the sulfonated azo compound 4-carboxy-4'-sulfoazobenzene as the sole source of carbon and energy. Appl. Environ. Microbiol. **64**: 2315-2317.

Boonchan, S., Britz, M.L., and Stanley, G.A. (2000). Degradation and mineralization of high molecular weight polycyclic aromatic hydrocarbons by defined fungal-bacterial coculcutures. Appl. Environ. Microbiol. **66**: 1007-1019.

Booparthy, R., and Kulper, C.F. (1993). Nitroaromatic compounds serve as nitrogen source for *Desulfovibrio* sp. (B strain). Curr. Microbiol. **39**: 430-433.

Booparthy, R., and Kulper, C.F. (1994). Biotransformation of 2,4,6-trinitrotoluene (TNT) by a *Methanococcus* sp. (strain B) isolated from a lake sediment. Can. J. Microbiol. **40**: 273-278.

Boris, J-R., Kannan, K., Addink, R., and Adams, D.H. (2005). Polychlorinated diphenyl ethers and polychlorinated biphenyls in a marine foodweb of coastal florida. Environ. Sci. Technol. **39**: 8243-8250.

Boyle, A.W., Silvin, C.J., Hassett, J.P., Nakas, J.P., and Tanenbaum, S.W. (1992). Bacterial PCB biodegradation. Biodegradation **3**: 285-298.

Braddock, J.F., and McCarthy, K.A. (1996). Hydrological and microbiological factors affecting persistence and migration of petroleum hydrocarbons spilled in a continuous-permafrost region. Environ. Sci. Technol. **30**: 2626-2633.

Bradford, M.M. (1976). A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72**: 248-254.

Bradley, P.M., and Chapelle, F.H. (1995). Rapid toluene mineralization by aquifer microorganisms at Adak, Alaska: implications for intrinsic bioremediation in cold environments. Environ. Sci. Technol. **29**: 2778-2781.

Braune, B.M., and Simon, M. (2003). Dioxins, furans, and non-ortho PCBs in Canadian Arctic seabirds. Environ. Sci. Technol. **37**: 3071-3077.

Bright, D.A., Dushenko, W.T., Grundy, S.L., and Reimer, K.J. (1995a). Evidence for short-range transport of polychlorinated biphenyls in the Canadian Arctic using congener signatures of PCBs in soils. Sci. Total Environ. **160/161**, 251-263.

Bright, D.A., Dushenko, W.T., Grundy, S.L., and Reimer, K.J. (1995b). Effects of local and

distant contaminant sources: polychlorinated biphenyls and other organochlorines in bottom-dwelling animals from an Arctic estuary. Sci. Total Environ. **160/161**, 265-283.

Broadus, R.M., and Haddock, J.D. (1998). Purification and characterization of the NADH: ferredoxin$_{BPH}$ oxidoreductase component of biphenyl 2,3-dioxygenase from *Pseudomonas* sp. strain LB400. Arch. Microbiol. **170**: 106-112.

Brown, J.F., Bedard, D.L., Brennan, M.J., Carnahan, J.C., Feng, H., and Wagner, R.E. (1987a). Polychlorinated biphenyl dechlorination in aquatic sediments. Science **236**: 709-712.

Brown, J.F., Wagner, R.E., Feng, H., Bedard, D.L., Brennan, M.J., Carnahan, J.C., and May, R.J. (1987b). Environmental dechlorination of PCBs. Environ. Toxicol. Chem. **5**: 579-593.

Brulhmann, F., and Chen, W. (1999). Transformation of polychlorinated biphenyls by a novel BphA variant through the *meta*-cleavage pathway. FEMS Microbiol. Lett. **179**: 203-208.

Brummer, I.H.M., Felske, A., and Wagner-Dobler, I. (2003). Diversity and seasonal variability of β-proteobacteria in biofilms of polluted rivers: analysis by temperature gradient gel electrophoresis and cloning. Appl. Environ. Microbiol. **69:** 4463-4473.

Buchan, A., Collier, L.S., Niedle, E.L., and Moran, M.A. (2000). Key aromatic-ring-cleaving enzyme, protocatechuate 3,4-dioxygenase, in the ecologically important marine *Roseobacter* lineage. Appl. Environ. Microbiol. **66**: 4662-4672.

Bull, A.T., Goodfellow, M., and Slater, J.H. (1992). Biodiversity as a source of innovation in biotechnology. Annu. Rev. Microbiol. **46**: 219-252.

Camara, B., Herrera, C., Gonzalez, M., Couve, E., Hofer, B., and Seegar, M. (2004). From PCBs to highly toxic metabolites by the biphenyl pathway. Environ. Microbiol. **6**: 842 – 850.

Chakraborty, R., and Coates, J.D. (2004). Anaerobic degradation of monoaromatic hydrocarbons. Appl. Microbiol. Biotechnol. **64**: 437-446.

Chan, M., Himes, R.H., and Akagi, J.M. (1971). Fatty acids composition of thermophilic, mesophilic, and psychrophilic clostridia. J. Bacteriol. **106**: 876-881.

Chapman, P.J. (1972). An outline of reaction sequences used for the bacterial degradation of phenolic compounds. In *Degradation of synthetic organic molecules in the biosphere.* pp. 17-55. Nat. Acad. Sci., Washington, DC, USA.

Chebrou, H., Hurtubise, Y., Barriault, D., and Sylvestre, M. (1999). Heterolous expression and characterization of the purified oxygenase component of *Rhodococcus globerulus* P6 biphenyl dioxygenase and of chimeras derived from it. J. Bacteriol. **181**: 4805-4811.

Chu, S., He, Y., and Xu, X. (1997). Determination of acute toxicity of polychlorinated biphenyls to *Photobacterium phosphoreum*. Bull. Environ. Contam. Toxicol. **58**: 263-267.

Chung, S.Y., Maeda, M., Song, E., Horikoshi, K., and Kudo, T. (1994). Isolation and characterization of a Gram-positive polychlorinated biphenyl-degrading bacterium, *Rhodococcus* erythropolis strain TA421, from a termite ecosystem. Biosci. Biotechnol. Biochem. **58**: 2111-2113.

Churchill, S.A., Harper, J.P., and Churchill, P.F. (1999). Isolation and characterization of a *Mycobacterium* species capable of degrading three- and four-ring aromatic and aliphatic hydrocarbons. Appl. Environ. Microbiol. **65**: 549-552.

Coates, J.D., and Lovely, D.R. (2003) Genus *Geobacter*. In *Bergey's Manual of Systematic Bacteriology*, eds. D. Brenner, N. Krieg, J. Staley, G. Garrity. Springer, New York, Berlin Heidelberg.

Contzen, M., Moore, E.R.B., Blumel, S., Stolz, A., and Kampfer, P. (2000). *Hydrogenophaga intermedia* sp. nov., a 4-aminobenzene-sulfonate degrading organism. Syst. Appl. Microbiol. **23**: 487-493.

Crawford, D.L. (1978). Lignocellulose decomposition by selected *Streptomyces* strains. Appl. Environ. Microbiol. **35**: 1041-1045.

Cutter, L.A., Watts, J.E.M., Sowers, K.R., and May, H.D. (2001). Identification of a microorganism that links its growth to reductive dechlorination of 2,3,5,6-chlorobiphenyl. Environ. Microbiol. **3**: 699-709.

Dagley, S. (1971). Catabolism of aromatic compounds by microorganisms. Adv. Microbiol. Physiol. **6**: 1-45.

Dagley, S. (1987). Lessons from biodegradation. Annu. Rev. Microbiol. **41**: 1-23.

Dai, S., Vaillancourt, F.H., Maarouff, H., Drouin, N.M., Neau, V.S., Bolin, J.T., and Eltis, L.D. (2002). Identification and analysis of a bottleneck in PCB biodegradation. Nat. Struct. Biol. **9**: 934-939.

Daniel, G., Nilsson, T., and Singh, A. (1987). Degradation of lignocellulose by unique tunnelling-forming bacteria. Can. J. Microbiol. **33**: 943-948.

Danse, I.R., Jaeger, R.J., Kava, R., Kroger, M., London, W.M., Lu, F.C., Maickel, R.P., McKetta, J.J., Newell, G.W., Shindell, S., Stare, F.J., and Whelan, E.M. (1997). Position paper of the American Council on Science and Public Health: Public health concern about polychlorinated biphenyls. Ecotoxicol. Environ. Saf. **38**: 71-84.

Deziel, E., Paquette, G., Villemur, R., Lepine, F., and Basaillon, J-G. (1996). Biosurfactant production by a soil pseudomonad strain growing on polycyclic aromatic hydrocarbons. Appl. Environ. Microbiol. **62**: 1908-1912.

Dmochewitz, S. and Ballschmiter, K. (1988). Microbial transformation of technical mixtures of polychlorinated biphenyl (PCB) by *Aspergillus niger*. Chemosphere. **17**: 111-121.

Dodge, R.H., Cerniglia, C.E., and Gibson, D.T. (1979). Fungal metabolism of biphenyl. Biochem. J. **128**: 123-130.

Dorn, E., and Knackmuss, H.-J. (1978). Chemical structure and biodegradability of halogenated aromatic compounds: two catechol 1,2-dioxygenase from a 3-chlorobenzoate-grown pseudomonad. Biochem. J. **174**: 73-84.

Drenzek, N.J., Eglinton, T.I., Wirsen, C.O., May, H.D., Wu, Q., Sowers, K.R., and Reddy, C.M. (2001). The absence and application of isotopic fractionation during reductive dechlorination of polychlorinated biphenyls. Environ. Sci. Technol. **35**: 310-313.

Duetz, W.A., Jong, C., Williams, P.A., Andel, J.G. (1994). Competition in chemostate culture between *Pseudomonas* strains that use different pathways for the degradation of toluene. Appl. Environ. Microbiol. **60**: 2858-2863.

Dunnivant, F.M., Elzerman, A.W., Jurs, P.C., and Hasan, M.N. (1992). Prediction of aqueous solubility and Henry's law constants for PCB congeners using quantitative structure-property relationships. Environ. Sci. Technol. **26**: 1567-1573.

Dutton, P.L., and Evans, W.C. (1969). The metabolism of aromatic compounds by *Rhodopseudomonas palustris*. Biochem. J. **113**: 525-535.

Dyksterhouse, S.E., Gray, J.P., Herwig, R.P., Lara, J.C., and Staley, J.T. (1995). *Cyclo-*

clasticus pugetii gen. nov., sp. nov., an aromatic hydrocarbon-degrading bacterium from marine sediments. Int. J. Sys. Bacteriol. **45**: 116-123.

Ehrlich, J. Bartz, Q.R., Smith, R.M., Joslyn, D.A., Burkholder, P.R. (1947). Chloromycetin: a new antibiotic from a soil actinomycete. Science **106**: 417

Environmental Agency, UK (2003). The determination of polychlorinated biphenyls by gas chromatography using mass spectrometric detection. Environmental Agency of the Government of the United Kingdom.

Environmental Sciences Group and Analytical Sciences Group, RMC and Analytical Services Unit, Queen's University (1996). Investigation of a PCB contamination at Saglek (LAB-2) long range radar site. Prepared for the North Warning System Office of the Department of National Defense (RMC-CCE-ES-96-11).

Erickson, M., Sodersten, E., Yu, Z., Dalhammar, G., and Mohn, W. (2003). Degradation of polycyclic aromatic hydrocarbons at low temperature under aerobic and nitrate-reducing conditions in enrichment cultures from northern soils. Appl. Environ. Microbiol. **69**: 275-284.

Erickson, M.D., Stanly, J.S., Turman, K., Radolovich, G., Bauer, K., Onstut, J., Rose, D., and Wickham, M. (1982). Analytical methods for PCBs by-product - preliminary validation and interim methods. Report EPA-50/5-82-006, NTIS no. PB 83 127 696. Office of Toxic Substances. U.S. Environmental Protection Agency, Washington DC

Evans, W.C. (1977). Biochemistry of bacterial catabolism of aromatic compounds in anaerobic environments. Nature. **270**: 17-22.

Fava, F., Gioia, D.D., Cinti, S., Marchetti, L., and Quattroni, G. (1994). Degradation and dechlorination of low-chlorinated biphenyls by a three-membered bacterial co-culture.

Appl. Microbiol. Biotechnol. **41**: 117-123.

Feigel, B.J., and Knackmuss, H.-J. (1993). Syntrophic interactions during degradation of 4-aminobenzenesulfonic acid by a two species bacterial culture. Arch. Microbiol. **159**: 124-130.

Feller, G. (2003). Molecular adaptations to cold in psychrophilic enzymes. Cell. Mol. Life Sci. **60**: 648-662.

Feller, G., and Gerday, C. (1997). Psychrophilic enzymes: molecular basis of cold adaptation. Cell Mol. Life Sci. **53**: 830-841.

Fetzner, S. (1998). Bacterial dehalogenation. Appl. Microbiol. Biotechnol. **50**: 633-657.

Flanagan, W.P., and May, R.J. (1993). Metabolite detection as evidence for naturally occurring aerobic PCB biodegradation in Hudson River Sediments. Environ. Sci. Technol. **27**: 2207-2212.

Frame, G.M., Cochrane, J.W., and Bowadt, S.S. (1996). Complete PCB congener distributions for 17 Aroclors mixtures determined by 3 HRGC systems optimized for comprehensive, quantitative, congener-specific analysis. High Resol. Chromatogr. **19**: 657-668

Franks, F., Mathias, S.F., and Hatley, R.H. (1990). Water, temperature and life. Phil. Trans. R. Soc. Lond. **B 326**, 517-533.

Fuenmayor, S.L., Wild, M., Boyes, A.L., and Williams, P.A. (1998). A gene cluster encoding the conversion of naphthalene to gentisate in *Pseudomonas* sp. strain U2. J. Bacteriol. **180**: 2522-2530.

Furukawa, K. (1994). Molecular genetics and evolutionary relationship of PCB-degrading bacteria. Biodegradation **5**:289-300.

Furukawa, K., and Arimura, N. (1987). Purification and properties of 2,3-dihydroxybiphenyl dioxygenase from polychlorinated biphenyl-degrading *Pseudomonas pseudoalcaligenes* and *Pseudomonas aeruginosa* carrying the cloned bphC gene. J. Bacteriol. **169**: 924-927.

Furukawa, K., Matsumura, F., and Tonomura, K. (1978). *Alcaligenes* and *Acinetobacter* strains capable of degrading polychlorinated biphenyls. Agric. Biol. Chem. **4**: 545-548.

Furukawa, K., Tomizuka, N., and Kamibayashi, A. (1979a). Effect of chlorine substitution on the bacterial metabolism of various polychlorinated biphenyls. Appl. Environ. Microbiol. **38**: 301-310.

Furukawa, K., Tonomura, K., and Kamibayashi, A. (1979b). Metabolism of 2,4,4'-trichlorobiphenyl by *Acinetobacter* sp. P6. Agric. Biol. Chem. **43**: 1577-1583.

Gibson, J., and Harwood, C. (2002). Metabolic diversity in aromatic compound utilization by anaerobic microbes. Annu. Rev. Microbiol. 2002. **56**: 345-369.

Gibson, D.T., and Parales, R.E. (2000). Aromatic hydrocarbon dioxygenases in environmental biotechnology. Curr. Opin. Biotechnol. **11**: 236-243.

Gibson, D.T., and Subramanian, V. (1984). Microbial degradation of aromatic hydrocarbons. In *Microbial degradation of organic compounds,* ed. D.T. Gibson. Mercel Dekker Inc. Chapter 7, pp. 182-252. New York, Basel.

Gibson, D.T., Roberts, R.L., Wells, M.C., Kobal, V.M. (1973). Oxidation of biphenyl by a *Beijerinckia* species. Biochem. Biophys. Res. Commun. **50**: 211-219.

Gibson, D.T., Mahadevan, V., and Davey, J.F. (1974). Bacterial metabolism of *para*- and *meta*-xylene: oxidation of the aromatic ring. J. Bacteriol. **119**: 930-936.

Gilbert, E.G., and Crowley, D. (1997). Plant compounds that induced polychlorinated bi-

phenyl biodegradation by *Arthrobacter* sp. strain B1B. Appl. Environ. Microbiol. **63**: 1933-1938.

Glazer, A. N., and Nikaido, H. (1995). Biomass. In *Microbial Biotechnology: Fundamental of Applied Microbiology,* W. H. Freeman and Company, New York.

Gopalakrishnan, J., Gorisch, and Adrian, L. (2003). Dehalorespiration with hexachlorobenzene and pentachlorobenzene by *Dehalococcoid* sp. strain BDBD1. Arch. Microbiol. **180**: 411-416.

Gribble, G.W. (1994). The natural production of chlorinated compounds. Environ. Sci. Technol. **28**: 310A-319A.

Gribble, G.W. (1996). Naturally occurring organohalogen compounds- a comprehensive survey. Proc. Org. Nat. Prod. **68**: 1-48.

Grund, E., Denecke, B., and Eichenlaub, R. (1992). Naphthalene degradation via salicylate and gentisate by *Rhodococcus* sp. strain B4. Appl. Environ. Microbiol. **58**: 1874-1877.

Gschwend, P.M., MacFarlane, J.K., and Newman, K.A. (1985). Volatile halogenated organic compounds released to seawater from temperate marine macroalgae. Science. **227**: 1033-1035.

Haddock, J.D., and Gibson, D.T. (1995). Purification and characterization of the oxygenase component of biphenyl 2,3-dioxygenase from *Pseudomonas* sp. strain LB400. J. Bacteriol **177**: 5834-5839.

Haddock, J.D., Nadim, L.M., and Gibson, D.T. (1993). Oxidation of biphenyl by a multicomponent enzyme system from *Pseudomonas* sp. strain LB400. J. Bacteriol. **175**: 395-400.

Haggblom, M.M. (1990). Mechanism of bacterial degradation and transformation of chlorinated nonaromatic compounds. J. Basic Microbiol. **32**: 115-141.

Hanne, L.F., Kirk, L.L., Appel, S.M., Narayan, A.D., and Bains, K.K. (1993). Degradation and induction specificity in actinomycetes that degrade *p*-nitrophenol. Appl. Environ. Microbiol. **59**: 3505-3508.

Harayama, S., Kok, M., and Neidle, E.L. (1992). Functional and evolutionary relationships among diverse oxygenases. Annu. Rev. Microbiol. **46**: 565-601.

Harkness, M.R., McDermott, J.B., and Abramowicz, D.A. (1993). In situ stimulation of aerobic degradation of PCB biodegradation in Hudson River sediments. Science **259**: 503-507.

Harwood, C.S., and Parales, R.E. (1996). The β-ketoadipate pathway and biology of self-identity. Ann. Rev. Microbiol. **50**: 553-90.

Hatakka, A. (1994). Lignin-modifying enzymes from selected white-rot fungi: production and role in lignin degradation. FEMS Microbiol. Rev. **13**: 125-135.

Hatzinger, P.B., McClay, K., Vainberg, S., Tugusheva, M., Condee, C.W., and Steffan, R.J. (2001). Biodegradation of methyl *tert*-butyl ether by a pure bacterial culture. Appl. Environ. Microbiol. **67**, 5601-5607.

Havel, J., and Reineke, W. (1991). Total degradation of various chlorobiphenyls by co-cultures and in vivo constructed hybrid pseudomonads. FEMS Microbiol. Lett. **78**: 163-170.

Havel, J., and Reineke, W. (1993). Microbial degradation of chlorinated acetophenones. Appl. Environ. Microbiol. **59**: 2706-2712.

Hickey, W.J., Brenner, V., and Focht, D.D. (1992). Mineralization of 2-chloro- and 2,5-

dichlorobiphenyl by *Pseudomonas* sp. strain UCR2. FEMS Microbiol. Lett. **98**: 175-180.

Higuchi, T. (1987). Catabolic pathways and role of ligninase for the degradation of lignin substructure model compounds by white-rot fungi. Wood Res. **73**: 58-81.

Hofrichter, M., Scheibner, K., Schneegab, I., and Fritsche, W. (1998). Enzymatic combustion of aromatic and aliphatic compounds by manganese peroxidase from *Nematoloma frowardii*. Appl. Environ. Microbiol. **64**: 399-404.

Holloman, T.R.P., Elberson, M.A., Cutter, L.A., May, H.D., and Sowers, K.R. (1998). Characterization of defined 2,3,5,6-tetrachlorobiphenyl-*ortho*-dechlorinating microbial community by comparative sequence analysis of genes coding for 16rRNA. Appl. Environ. Microbiol. **64**: 3359-3367.

Hung, H., Halsall, C.J., Blanchard, P., Li, H.H., Fellin, P., Stern, G., and Rosenberg, B. (2001). Are PCBs in the Canadian Arctic atmosphere declining? Evidence from 5 years of monitoring. Environ. Sci. Technol. **35**: 1303-1311.

Hurtubise, Y., Barriault D., Powlowski, J., and Sylvestre, M. (1995). Purification and characterization of the *Comamonas testosteroni* B-356 biphenyl dioxygenase components. J. Bacteriol. **177**: 6610-6618.

Hurtubise, Y., Barriault, D., and Sylvestre, M. (1998). Involvement of the terminal oxygenase β subunit in the biphenyl dioxygenase reactivity pattern toward chlorobiphenyls. J. Bacteriol. **180**: 5828-5835.

Hutzinger, O., Safe, S., and Zitko, V. (1974). The Chemistry of PCBs. CRC Press, Cleveland, Ohio.

Hrywna, Y., Tsoi, T.V., Maltseva, O.V, Quensen III, J.F., and Tiedje, J.M. (1999). Con-

struction and characterization of two recombinant bacteria that grow on *ortho*- and *para*-substituted chlorobiphenyls. Appl. Environ. Microbiol. **65**: 2163-2169.

Imamoglu, I., Li, K., Christensen, E.R., and McMullin, J.K. (2004). Sources and dechlorination of polychlorinated biphenyl congeners in the sediments of Fox River, Wisconsin. Environ. Sci. Technol. **38**: 2574-2583.

Imbeault, N.Y.R., Powlowski, J.B., Colbert, C.L., Bolin J.T., and Eltis, L.D. (2000). Steady-state kinetic characterization and crystallization of a polychlorinated biphenyl-transforming dioxygenase. J. Biol. Chem. **275**: 12430-12437.

Janssen, D.B., Pries, F., and van der Ploeg, J.R. (1994). Genetics and biochemistry of dehalogenating enzymes. Annu. Rev. Microbiol. **48**: 163-191.

Jarvinen, K.T., Melin, E.S., and Puhakka, J.A. (1994). High-rate bioremediation of chlorophenol-contaminated groundwater at low temperatures. Environ. Sci. Technol. **28**: 2387-2392.

Jerina, D.M., and Daly, J.W. (1974). Arene oxides: A new aspect of drug metabolism. Science. **185**: 573-582.

Jong, E.D., Field, J.A., and Bont, J.A.M. (1994a). Aryl alcohols in the physiology of ligninolytic fungi. FEMS Microbiol. Rev. **13**: 153-188.

Jong, E.D., Field, J.A., Spinnler, H.E., Wijnberg, J.B.P.A., and Bont, J.A.M. (1994b). Significant biogenesis of chlorinated aromatics by fungi in natural environments. Appl. Environ Microbiol. **60**: 264-270.

Joshi, B., and Walia, S. (1995). Characterization by arbitrary primer polymerase chain reaction of polychlorinated biphenyl (PCB)-degrading strains of *Comamonas testosteroni* isolated from PCB-contaminated soil. Can. J. Microbiol. **41**: 612-619.

Kampfer, P., Schulze, R., Jackel, U., Malik, K.A., Amann, R., and Spring, S. (2005). *Hydrogenophaga defluvii* sp. nov. and *Hydrogenophaga atypical* sp. nov., isolated from activated sludge. Int. J. Sys. Evol. Microbiol. **55:** 341-344.

Kassen, R., and Rainey, P.B. (2004). The ecology and genetics of microbial diversity. Annu. Rev. Microbiol. **58**: 207-231.

Kato, T., Haruki, M., Imanaka, T., Morikawa, M., and Kanaya, S. (2001). Isolation and characterization of psychrotrophic bacteria from oil-reservoir water and oil sands. Appl. Microbiol. Biotechnol. **55**: 794-800.

Kersten, P.J. (1990). Glyoxal oxidase of *Phanerochaete chrysosporium*: its characterization and activation by lignin peroxidase. Proc. Natl. Acad. Sci. USA **87**: 2936-2940.

Khan, A., and Walia, S. (1989). Cloning of bacterial genes specifying degradation of 4-chlorobiphenyl from *Pseudomonas putida* OU83. Appl. Environ. Microbiol. **55**: 798-805.

Kikuchi, Y., Nagata, Y., Hinata, M., Kimbara, K., Fukuda, M., Yano, K., and Takagi, M. (1994). Identification of the bphA4 Gene encoding ferredoxin reductase involved in biphenyl and polychlorinated biphenyl degradation in *Pseudomonas* sp. strain KK102. J. Bacteriol. **176**: 1689-1694.

Kim, S., and Picardal, F. (2000). A novel bacterium that utilizes monochlorobiphenyls and 4-chlorobenzoate as growth substrates. FEMS Microbiol. Lett. **185**: 225-229.

Kim, S., and Picardal, F. (2001). Microbial growth on dichlorobiphenyls chlorinated on both rings as a sole carbon and energy source. Appl. Environ. Microbiol. **67**: 1953-1955.

King, G.M. (1986). Inhibition of microbial activity in marine sediments by a bromophe-

nol from a hemichordate. **323**: 257-259.

Kirk, T.K. (1984). Degradation of lignin. In *Microbial Degradation of Organic Compounds,* ed. D.T. Gibson, pp. 399-437. Dekker, New York.

Kirk, T.K., and Farrell, R.L. (1987). Enzymatic "combustion": The microbial degradation of lignin. Annu. Rev. Microbiol. **41**: 465-505.

Knoll, G., and Winter, J. (1989). Degradation of phenol via carboxylation to benzoate by a defined, obligate syntrophic consortium of anaerobic bacteria. Appl. Microbiol. Biotechnol. **30**: 318-324.

Kohler, H.-P.E., Kohler-Staub, D., and Focht, D.D. (1988). Cometabolism of polychlorinated biphenyls: Enhanced transformation of Aroclor 1254 by growing bacteria cells. Appl. Environ. Microbiol. **54**: 1940-1945.

Kotturi, G., Robinson, C.G., and Innis, W.E. (1991). Phenol degradation by a psychotropic strain of *Pseudomonas putida.* Appl. Microbiol. Biotechnol. **34**: 539-543.

Kuipers, B., Cullen, W.R., and Mohn, W.W. (2003). Reductive dechlorination of weathered Aroclor 1260 during anaerobic biotreatment of Arctic soils. Can. J. Microbiol. **49**: 9-14.

Lambo, A.J., and Patel, T.R. (2006a). Isolation and characterization of a biphenyl-utilizing psychrotolerant bacterium, *Hydrogenophaga taeniospiralis* IA3-A, that cometabolize dichlorobiphenyls and polychlorinated biphenyl congeners in Aroclor 1221. J. Basic Microbiol. **46**: 94-107.

Lambo, A.J., and Patel, T.R. (2006b). Cometabolic degradation of polychlorinated biphenyls at low temperature by psychrotolerant bacterium *Hydrogenophaga* sp. IA3-A. Curr. Microbiol. **53**: 48-52.

Layton, A.C., Sanseverino, J., Wallace, Corcoran, C., and Sayler, G.S. (1992). Evidence for

4-chlorobenzoic dehalogenation mediated by plasmids related to pSS50. Appl. Environ. Microbiol. **58**: 399-402.

Lindner, A.S., Adriaens, P., and Semrau, J.D. (2000). Transformation of *ortho*-substituted biphenyls by *Methylosinus trichosporium* OB3b: substituents effects on oxidation kinetics and products formation. Arch. Microbiol. **174**: 35-41.

Lovely, D.R., Baedecker, M.J., Lonergan, D.J., Cozzarelli, I.M., Phillips, E.J.P., and Siegel, D.I. (1989) Oxidation of aromatic contaminants coupled to microbial iron reduction. Nature. 339: 297-300.

Ma, Y., Wang, L., and Shao, Z. (2006). *Pseudomonas*, the dominant polycyclic aromatic hydrocarbon-degrading bacteria isolated from Antarctic soils and the role of large plasmids in horizontal gene transfer. Environ. Microbiol. **8**: 455-465.

Maeda, M., Chung, S.-Y., Song, E., and Kudo, T. (1995). Multiple genes encoding 2,3-dihydroxybiphenyl 1,2-dioxygenase in the gram-positive polychlorinated biphenyl-degrading bacterium *Rhodococcus erythropolis* TA421, isolated from a termite eco-system. Appl. Environ. Microbiol. **61**: 549-555.

Magar, V.S., Brenner, R.C., Johnson, G.W., and Quensen, J.F. (2005). Long-term recovery of PCB-contaminated sediments at the Lake Hartwell superfund site: PCB dechlorination. 2. Rates and extent. Environ. Sci. Technol. **39**: 3548-3554.

Maltseva, V.O., Tsoi, T.V., Quensen III, J.F., Fukuda, M., and Tiedje, J.M. (1999). Degradation of anaerobic reductive dechlorination products of Aroclor 1242 by four aerobic bacteria. Biodegradation **10**: 363-371.

Mannisto, M.K., Tiirola, M.A., and Puhakka, J.A. (2001). Degradation of 2,3,4,6-tetrachlorophenol at low temperature and low dioxygen concentrations by phylogenetically different

groundwater and bioreactor bacteria. Biodegradation **12**: 291-301.

Margesin, R., Labbe, D., Schinner, F., Greer, C.W., and Whyte, L.G. (2003). Characterization of hydrocarbon-degrading microbial populations in contaminated and pristine Alpine soils. Appl. Environ. Microbiol. **69**: 3085-3092.

Margesin, R., and Schinner, F. (1998a). Oil biodegradation potential in Alpine Habitats. Arct. Alp. Res. **30**: 262-265.

Margesin, R., and Schinner, F. (1998b). Low-temperature bioremediation of a waste water contaminated anionic surfactants and fuel oil. Appl. Microbiol. Biotechnol. **49**: 482-486.

Masse, R., Messier, F., Peloquin, L., Ayotte, C., and Sylvestre, M. (1984). Microbial biodegradation of 4-chlorobiphenyl, a model compound of chlorinated biphenyls. Appl. Environ. Microbiol. **47**: 947-951.

Masse, R., Messier, F., Ayotte, C., Levesque, M-F., and Sylvestre, M. (1989). A comprehensive gas chromatographic/mass spectrometric analysis of 4-chlorobiphenyl bacterial degradation products. Biomed. Environ. Mass Spectromet. **18**: 27-47.

Master, E.R., Lai, V.W.M., Kuipers, B., Cullen, W.R., and Mohn, W.W. (2002). Sequential anaerobic-aerobic treatment of soil contaminated with weathered Aroclor 1260. Environ. Sci. Technol. **36**: 100-103.

Master, E.R., and Mohn, W.W. (1998). Psychrotolerant bacteria isolated from Arctic soil that degrade polychlorinated biphenyls at low temperatures. Appl. Environ. Microbiol. **64**: 4823-4829.

Master, E.R., and Mohn, W.W. (2001). Induction of bphA, encoding biphenyl dioxygenase, in two polychlorinated biphenyl-degrading bacteria, psychrotolerant *Pseudomonas* strain Cam-1 and mesophilic *Burkholderia* strain LB400. Appl. Environ. Microbiol.

67: 2669–2676

McCarthy, D.L., Louie, D.F., and Copley, S.D. (1997). Identification of a covalent intermediate between gluthatione and cysteine13 formed during catalysis by tetrachloroquinone dehalogenase. J. Am. Chem. Soc. **119**: 11337-11338.

McCullar, M.V., Brenner, V., Adams, R.H., and Focht, D.D. (1994). Construction of a novel PCB-degrading bacterium- utilization of 3,4-dicholorobiphenyl by *Pseudomonas* M3GY. Appl. Environ. Microbiol. **60**: 3833-3839.

Meredith, M.L., and Hites, R.A. (1987). Polychlorinated biphenyl accumulation in tree bark and wood growth rings. Environ. Sci. Technol. **21**: 709-712.

Miyaushi, K., Suh, S-K., Nagata, Y., Takagi, M. (1998). Cloning and sequencing of a 2,5-dichlorohydroquinone reductive dehalogenase gene whose product is involved in the degradation of γ-hexachlorocyclohexane by *Sphingomonas paucimobilis*. J. Bacteriol. **180**: 1354-1359.

Mohn, W.W., and Tiedje J.M. (1992). Microbial reductive dehalogenation. Microbiol. Rev. **56**: 482-507.

Mohn, W.W., Westerberg, K., Cullen, W.R., and Reimer, K.J. (1997). Aerobic biodegradation of biphenyl and polychlorinated biphenyls by Arctic soil microorganisms. Appl. Environ. Microbiol. **9**: 3378-3384

Mondello, F.J. (1989). Cloning and expression in *Escherichia coli* of *Pseudomonas* strain LB400 genes encoding polychlorinated biphenyl degradation. J. Bacteriol. **171**: 1725-1732.

Mondello, F.J., Turcich, M.P., Lobos, J.H., and Erickson, B.D. (1997). Identification and modification of biphenyl dioxygenase sequences that determine the specificity of

polychlorinated biphenyl degradation. Appl. Environ. Microbiol. **63**: 3096-3103

Morita, R.Y. (1975). Psychrophilic bacteria. Bacteriol. Rev. **39**: 144-167.

Muheim, A., Waldner, R., Leisola, M.S.A, and Fiechter, A. (1990). An extracellular aryl-alcohol oxidase enzyme from the white-rot fungus *Bjerkandera adusta*. Enz. Microb. Technol. **12**: 204-209.

Mullin, M.D., Pochini, C.M., McCrindle, S., Romkes, M., Safe, S.H. and Safe, L.M. (1984). High-resolution PCB analysis: synthesis and chromatographic properties of all 209 PCB congeners. Environ. Sci. Technol. **18**, 468-476.

Narro, M.L., Cerniglia, C.E., Baalen, C.V., and Gibson, D.T. (1992). Evidence of an NIH shift in oxidation of naphthalene by the marine cyanobacterium *Oscillatoria* sp. strain JCM. Appl. Environ. Microbiol. **58**: 1360-1363.

Nedwell, D.B., and Rutter, M. (1994). Influence of temperature on growth rate and competition between two psychrotolerant Antarctic bacteria: low temperature diminishes affinity for substrate uptake. Appl. Environ. Microbiol. **60**: 1984-1992.

Nies, L., and Vogel, T.M. (1990). Effects of organic substrates on dechlorination of Aroclor 1242 in anaerobic sediments. Appl. Environ. Microbiol. **56**: 2612-2617.

Oberg, G. (2002). The natural chlorine cycle: fitting the scattered pieces. Appl. Microbiol. Biotechnol. **58**: 565-581.

Ogunseitan, O.A. (2005). Microbial Diversity. Blackwell Publishing, Oxford, England.

Ornston, L.N. (1966). The conversion of catechol and protocatechuate to β-ketoadipate by *Pseudomonas putida*. J. Biol. Chem. **241**: 3800-3810.

Ornston, M.K., and Ornston, L.N. (1966). The regulation of the β-ketoadipate pathway in *Pseudomonas acidivorans* and *Pseudomonas testosteroni*. J. Gen. Microbiol. **73**:

455-464.

Patel, T.R., and Bartlett, F.M. (1988). Heat stable proteases from psychrotrophic pseudomonads: secondary structure and heat stability. Food Microbiol. **5**: 201-211.

Pieper, D. (2005). Aerobic degradation of polychlorinated biphenyls. Appl. Microbiol. Biotechnol. **67**: 170-191.

Pereira, W.E., Rostad, C.E., and Taylor H.E. (1980). Mount St. Helens, Washington, 1980 volcanic eruption: Characterization of organic compounds in ash samples. Geophy. Res. Lett. **7**: 953-954.

Potrawfke, T., Lohnert, T.H., Timmis, K.N., and Wittich, R.-M. (1998). Mineralization of low-chlorinated biphenyls by *Burkholderia* sp. strain LB400 and by a two-membered consortium upon directed interspecies transfer of chlorocatechol pathway genes. Appl. Microbiol. Biotechnol. **50**: 440-446.

Prenafeta-Boldu, F.X., Vervoort, J., Grotenhuis, J.T.C., and van Groenestijn, J.W. (2002). Substrate interactions during biodegradation of benzene, toluene, ethylbenzene, and xylenes (BTEX) hydrocarbon by fungus *Cladophialophora* sp. strain T1. Appl. Environ. Microbiol. **68**: 2660-2665.

Quensen III J.F., and Tiedje, J.M. (1998). Evaluation of PCB dechlorination in sediments. In *Methods in Biotechnology*, ed. D. Sheehan, Vol. 2, pp. 257-273. Bioremediation Protocol. Humana Press, Inc., Totowa, New Jersey.

Quensen, J.F. III, Tiedje, J.M., and Boyd, S.A. (1988). Reductive dechlorination of polychlorinated biphenyls by anaerobic microorganisms from sediments. Science **242**: 752-754.

Reineke, W. (1984). Microbial degradation of halogenated aromatic compounds. In

Microbial Degradation of Organic Compounds, ed. D.T. Gibson, Chapter 11, pp. 319-360. Marcel Dekker Inc., New York, Basel.

Reineke, W., and Knackmuss, H.J. (1988). Microbial degradation of haloaromatics. Annu. Rev. Microbiol. **42**: 262-287.

Robinson, G.K., and Lenn, M.J. (1994). The bioremediation of polychlorinated biphenyls (PCBs): problems and perspectives. Biotechnol. Gene. Eng. Rev. **12**: 139-188.

Romanov, V., and Hausinger, R. (1994). *Pseudomonas aeruginosa* 142 uses a three-component *ortho*-halobenzoate 1,2-dioxygenase for metabolism of 2,4-dichloro- and 2-chlorobenzoate. J. Bacteriol. **176**: 3368-3374.

Russell, N.J. (1984). Mechanisms of thermal adaptation in bacteria: blueprints for survival. Trends Biochem. Sci. **9**: 108-112.

Russell, N.J., Harrisson, P., Johnston, I.A., Jaenicke, R., Zuber, M., Franks, F., and Wynn-Williams, D. (1990). Cold adaptation of microorganisms. Phil. Trans. R. Soc. Lond. **326**: 595-611.

Rutter, M., and Nedwell, D. B. (1994). Influence of changing temperature on growth rate and competition between two psychrotolerant Antarctic bacteria: competition and survival in non-steady state temperature environments. Appl. Environ. Microbiol. **60**: 1993-2002.

Rybkina, D.O., Plotnikova, E.G., Dorofeeva, L.V., Mironenka, Y.L., and Demakov (2003). A new aerobic Gram-positive bacterium with a unique ability to degrade *ortho*- and *para*-chlorinated biphenyls. Microbiology **72**: 672-677.

Sannia, G., Limongi, P., Cocca, E., Buonocore, F., Nitti, G., and Giardina, P. (1991). Purification and characterization of a veratyl alcohol oxidase enzyme from the lignin degrading basidiomycete *Pleurotus ostreatus*. Biochim. Biophys. Acta **1073**: 114-

119.

Sasek, V., Volfova, O., Erbanova, P., Vyas, B.R.M., and Matucha, M. (1993). Degradation of PCBs by white rot fungi, methylotrophic, and hydrocarbon utilizing yeast and bacteria. Biotech. Lett. **15**: 521-526.

Satoh, A.Y., Trosko, J.E., and Masten, S.J. (2003). Epigenetic toxicity of hydroxylated biphenyls and polychlorinated biphenyls on a normal rate liver epithelial cell. Environ. Sci. Technol. **37**: 2727-2733.

Schwein, U., Schmidt, E., Knackmuss, H.-J., and Reineke, W. (1988). Degradation of chloro-substituted aromatic compounds by *Pseudomonas* sp. strain B13: fate of 3,5-dichloro-catechol. Arch. Microbiol. **150**: 78-84

Seah, S.Y.K., Labbe, G., Kaschabek, S.R., Reifenrath, F., Reineke, W., Eltis, L.D. (2001). Comparative specificities of two evolutionary divergent hydrolases involved in microbial degradation of polychlorinated biphenyls. J. Bacteriol. **183**: 1511-1516.

Seah, S.Y.K., Labbe, G., Nerdinger, S., Johnson, M.R., Snieckus, V., and Eltis, L.D. (2000). Identification of a serine hydrolase as a key determinant in the microbial degradation of polychlorinated biphenyls. J. Biol. Chem. **275**: 15701-15708.

Seeger, M., Timmis, K.N., and Hofer, B. (1995). Conversion of chlorobiphenyls into phenylhexanoates and benzoates by the enzymes of the upper pathway for polychlorinated degradation encoded by the *bph* locus of *Pseudomonas* sp. strain LB400. Appl. Environ. Microbiol. **61**: 2654-2658.

Seeger, M., Timmis, K.N., and Hofer, B. (1997). Bacterial pathways for the degradation of polychlorinated biphenyls. Mar. Chem. **58**: 327-333.

Semple, K.T., and Cain, R.B. (1996). Biodegradation of phenols by the alga *Ochromonas*

danica. Appl. Environ. Microbiol. **62**: 1265-1273.

Seto, M., Kimbara, K., Shimura, M., Hatta, T., Fukuda, M., and Yano, K. (1995). A novel transformation of polychlorinated biphenyls by *Rhodococcus* sp. strain RHA1. Appl. Environ. Microbiol. **61**: 3353-3358.

Singh, A., Nilsson, T., and Daniel, G. (1987). Ultrastructure of the attack of the wood of two high lignin tropical hardwood species, *Alstonai scholaris* and *Homalium foetium*, by tunnelling bacteria. J. Inst. Wood Sci. **11**: 26-42.

Siron, R., Pelletier, E., and Brochu, C. (1995). Environmental factors influencing the biodegradation of petroleum hydrocarbons in cold seawater. Arch. Environ. Contam. Toxicol. **28**: 406-416.

Smidt, H., and de Vos, W.M. (2004). Anaerobic microbial dehalogenation. Annu. Rev. Microbiol. **58**: 43-73.

Smith, M.R., and Ratledge, C. (1989). Catabolism of biphenyl by *Pseudomonas* sp. NCIB 10643 and *Nocordia* sp. NC1B 10503. Appl. Microbiol. Biotech. **30**: 395-401.

Sondossi, M., Sylvestre, M., and Ahmad, D. (1992). Effects of chlorobenzoate transformation on the *Pseudomonas testosteroni* biphenyl and chlorobiphenyl degradation pathway. Appl. Environ. Microbiol. **58**: 485-495.

Sotosky, J.B., and Atlas, R.M. (1994). Frequency of genes in aromatic and aliphatic hydrocarbon biodegradation pathways within bacterial populations from Alaskan sediments. Can. J. Microbiol. **40**: 981-985.

Spain, J.C. (1995). Biodegradation of nitroaromatic compounds. Annu. Rev. Microbiol. **49**: 523-555.

Stanier, R.Y., and Ornston, L.N. (1973). The β-ketoadipate pathway. Adv. Microbial

Physiol. **9**: 89-151.

Stringfellow, W.T., and Aitken, M.D. (1995). Competitive metabolism of naphthalene, methylnaphthalene, and fluorene by phenanthrene-degrading *Pseudomonads*. Appl. Environ. Microbiol. **61**: 357- 362.

Subramanian, V., Liu, T.-N., Yeh, W.-K., Narro, M., and Gibson, D.T. (1981). Purification and properties of NADH-ferrodoxin$_{TOL}$ reductase. A component of toluene dioxygenase from *Pseudomonas putida*. J. Biol. Chem. **256**: 2723-2730.

Subramanian, V., Liu, T.-N., Yeh, W.-K., Serdar, C.M., Wackett, P.L., and Gibson, D.T. (1985). Purification and properties of ferrodoxin$_{TOL}$. A component of toluene dioxygenase from *Pseudomonas putida* F1. J. Biol. Chem. **260**: 2355-2363.

Suenaga, H., Watanabe, T., Sato, M., Ngadima, and Furukawa, K. (2002). Alteration of regiospecificity in biphenyl dioxygenase by active-site engineering. J. Bacteriol. **184**: 3682-3688.

Sutherland, J.B., Freeman, J.P., Selby, A.L., Fu, P.P., Miller, D.W., and Cerniglia, C.E. (1990). Stereoselective formation of a K-region dihydrodiol from phenanthrene by *Streptomyces flavovirens*. Arch. Microbiol. **154**: 260-266.

Taylor, B.F., Campbell, W.L., and Chinoy, I. (1970). Anaerobic degradation of benzenoid nucleus by a facultatively anaerobic microorganism. J. Bacteriol. **102**: 430-437.

Tejedor, M.C., Murado, M.A., and Baluja, G. (1979). Oxidative metabolism in *Saccharomyces cerevisiae* as affected by polychlorinated biphenyls. Bull. Environ. Contam. Toxicol. **22**: 439-448.

Tiirola, M.A., Busse, H.-J., Kampfer, P., and Mannisto, M.K. (2005). *Novosphingobium lentum* sp. nov., a psychrotolerant bacterium from a polychlorophenol bioreme-

diation process. Int. J. Sys. Evol. Microbiol. **55**: 583-588.

Timmis, K.N., Steffan, R.J., and Unterman, R. (1994). Designing microorganisms for the treatment of toxic wastes. Annu. Rev. Microbiol. **48**: 525-557.

Tsitko, I.V., Zaitsev, G.M., Lobanok, A.G., and Salkinoja-Salonen, M.S. (1999). Effect aromatic compounds on fatty acid composition of *Rhodococcus opacus*. Appl. Environ. Microbiol. **65**: 853-855.

Tucker, E.S., Saeger, V.W., and Hicks, O. (1975). Activated sludge primary degradation of polychlorinated biphenyls. Bull. Environ. Contam. Toxicol. **14**: 705-712.

van Dort, H.M., and Bedard, D.L. (1991). Reductive *ortho* and *meta* dechlorination of polychlorinated biphenyl congener by anaerobic microorganisms. Appl. Environ. Microbiol. **57**, 1576-1578.

van Pee, K.H. (1996). Biosynthesis of halogenated metabolites by bacteria. Annu. Rev. Microbiol. **50**: 375-399.

vanden Hoven, R.N., and Santini, J.M. (2004). Arsenite oxidation by the heterotroph *Hydrogenophaga* sp. strain NT-14: the arsenite oxidase and its physiological electron acceptor. Biochim. Biophys. Acta **1656**: 148-55.

Vanderberg, L.A., Krieger-Grumbine, R., and Taylor, M.N. (2000). Evidence for diverse oxidations in the catabolism of toluene by *Rhodococcus rhodochrous* strain OFS. Appl. Microbial. Biotechnol. **53**: 447-452.

Vargas, C., Song, B., Camps, M., and Haggblom, M.M. (2000). Anaerobic degradation of fluorinated aromatic compounds. Appl. Microbiol. Biotechnol. **53**: 342-347.

Vicuna, R. (1988). Bacterial degradation of lignin. Enzyme Microb. Technol. **10**: 645-655.

Vogel, T.M., and Grbic-Galic, D. (1986). Incorporation of oxygen from water into toluene and benzene during anaerobic fermentative transformation. Appl. Environ. Microbiol. **52**: 200-202.

Vrana, B., Dercova, K., and Bala, S. (1996). Evaporation kinetics of polychlorinated biphenyls during biodegradation experiments. Biotechnol. Tech. **10**: 37-40.

Whyte, L.G., Bourbonniere, L., and Greer, C.W. (1997). Biodegradation of petroleum hydrocarbons by psychrotrophic *Pseudomonas* strains possessing both (*alk*) and naphthalene (*nah*) catabolic pathways. Appl. Environ. Microbiol. **63**: 3719-3723.

Whyte, L.G., Greer, C.W., and Inniss, W.E. (1995). Assessment of the biodegradation potential of psychrotrophic microorganisms. Can. J. Microbiol. **42**: 99-106.

Wicklow, D.T., Langie, R., Crabtree, S., and Detroy, R.W. (1984). Degradation of lignocellulose in wheat straw versus hardwood by *Cyathus* and related species (*Nidularia ceae*). Can. J. Microbiol. **30**: 632-636.

Willems, A., Busse, J., Goor, M., Pot, B., Falsen, E., Jantzen, E., Hoste, B., Gillis, M., Kersters, K., Auling, G., and Deley, J. (1989) *Hydrogenophaga*, a new genus of hydrogen-oxidizing bacteria that includes *Hydrogenophaga flava* comb. nov. (formerly *Pseudomonas flava*), *Hydrogenophaga palleronii* (formerly *Pseudomonas palleronii*), *Hydrogenophaga pseudoflava* (formerly *Pseudomonas pseudoflava* and *Pseudomonas carboxydoflava*) and *Hydrogenophaga taeniospiralis* (formerly *Pseudomonas taeniospiralis*). Int. J. Syst. Bacteriol. **39**: 319-333.

Williams, W.A., Lobos, J.H. and Cheetham, W.E. (1997). A phylogenetic analysis of aerobic polychlorinated biphenyl-degrading bacteria. Int. J. Syst. Bacteriol. **47**: 207-210.

Williams, W.A., and May, R.J. (1997). Low-temperature microbial aerobic degradation of polychlorinated biphenyls in sediment. Environ. Sci. Technol. **31**: 3491-3496.

Wright, G.E., and Madigan, M.T. (1991). Photocatabolism of aromatic compounds by phototrophic purple bacterium *Rhodomicrobium vanielli*. Appl. Environ. Microbiol. **57**: 2069-2073.

Wu, Q., Watts, J.E.M., Sowers, K.R., and May, H.D. (2002). Identification of a bacterium that specifically catalyzes the reductive dechlorination of polychlorinated biphenyl with double flanked chlorines. Appl. Environ. Microbiol. **68**: 807-812.

Wunder, T., Kremer, S., Sterner, O., and Anke, H. (1994). Metabolism of polycyclic aromatic hydrocarbon pyrene by *Aspergillus niger* SK 9317. Appl. Microbiol. Biotech. **42**: 636-641.

Xun, L., and Orser, C.S. (1991). Purification and properties of pentachlorophenol hydroxylase, a flavoprotein from *Flavobacterium* sp. strain ATCC 39723. J. Bacteriol. **173**: 4447-4453.

Yadav, J.S., Quensen III, J.F., Tiedje, J.M., and Reddy, C.A. (1995). Degradation of polychlorinated biphenyl mixtures (Aroclor 1242, 1254, 1260) by white-rot fungus *Phanerochaete chrysosporium* as evidenced by congener-specific analysis. Appl. Environ. Microbiol. **61**: 2560-2565.

Yadav, J.S., and Reddy, C.A. (1993). Degradation of benzene, toluene, ethylbenzene, and xylene (BTEX) by lignin-degrading basidiomycete *Phanerochaete chrysosporium.* Appl. Environ. Microbiol. **59**: 756-762.

Yeh, M.-F., and Hong, C.-H. (2002). Octanol-water partition coefficients of non-*ortho* and mono-*ortho*-substituted polychlorinated biphenyls. J. Chem. Eng. Data. **47**: 209-

215.

Yumoto, I., Hirota, K., Iwata, H., Akutsu, M., Kusumoto, K., Morita, N., Ezura, Y., Okuyama, H., and Matsuyama, H. (2004). Temperature and nutrient availability control growth rate and fatty acid composition of facultative psychrophilic *Cobetia marina* strain L-2. Arch. Microbiol. **181**: 345-351.

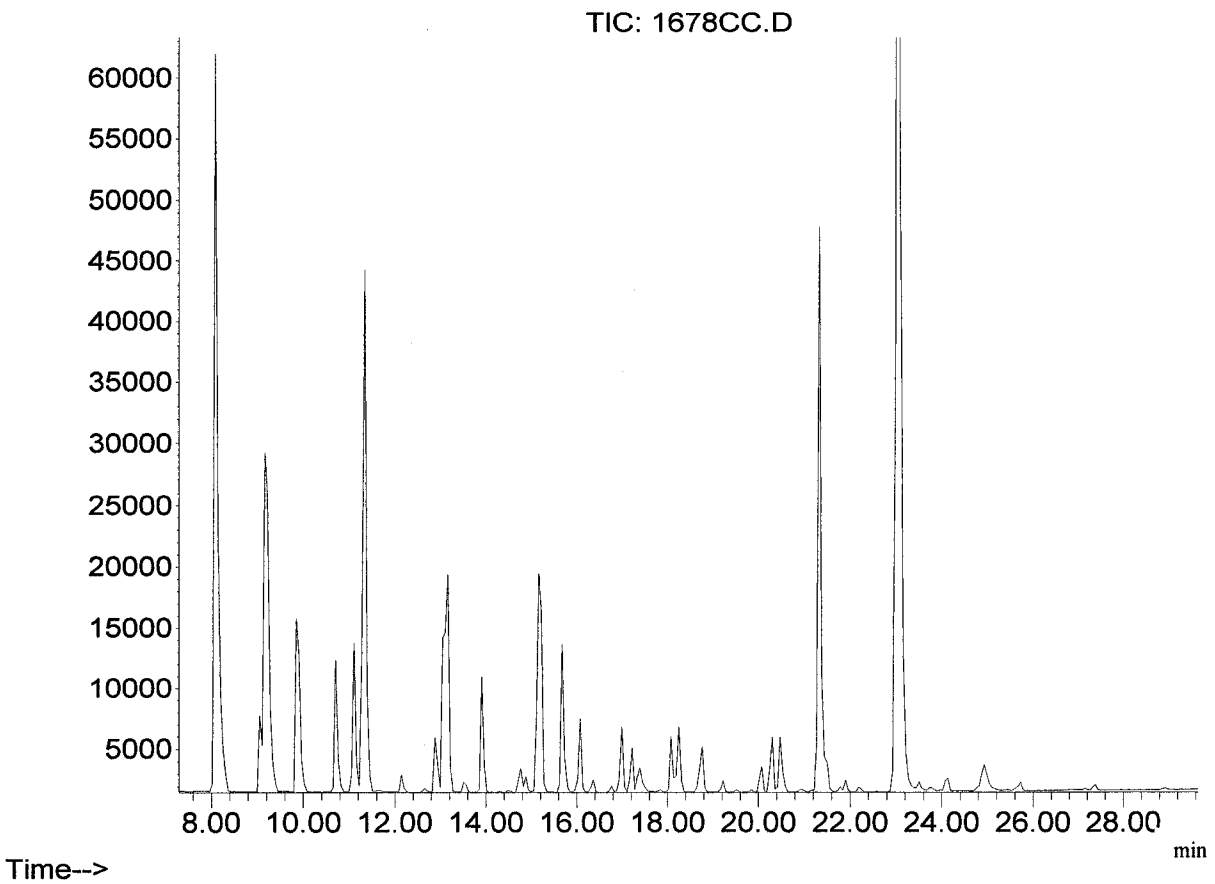
Zhang, X., and Young, L.Y. (1997). Carboxylation as an initial reaction in the anaerobic metabolism of naphthalene and phenanthrene by sulfidogenic consortia. Appl. Environ. Microbiol. **63**: 4759-4764.

Zhao, S., Narang, A., Ding, X., and Eadon. G. (2004). Characterization and quantitative analyses of DNA adducts formed from lower chlorinated PCB-derived quinones. Chem. Res. Toxicol. **17**: 502-511.

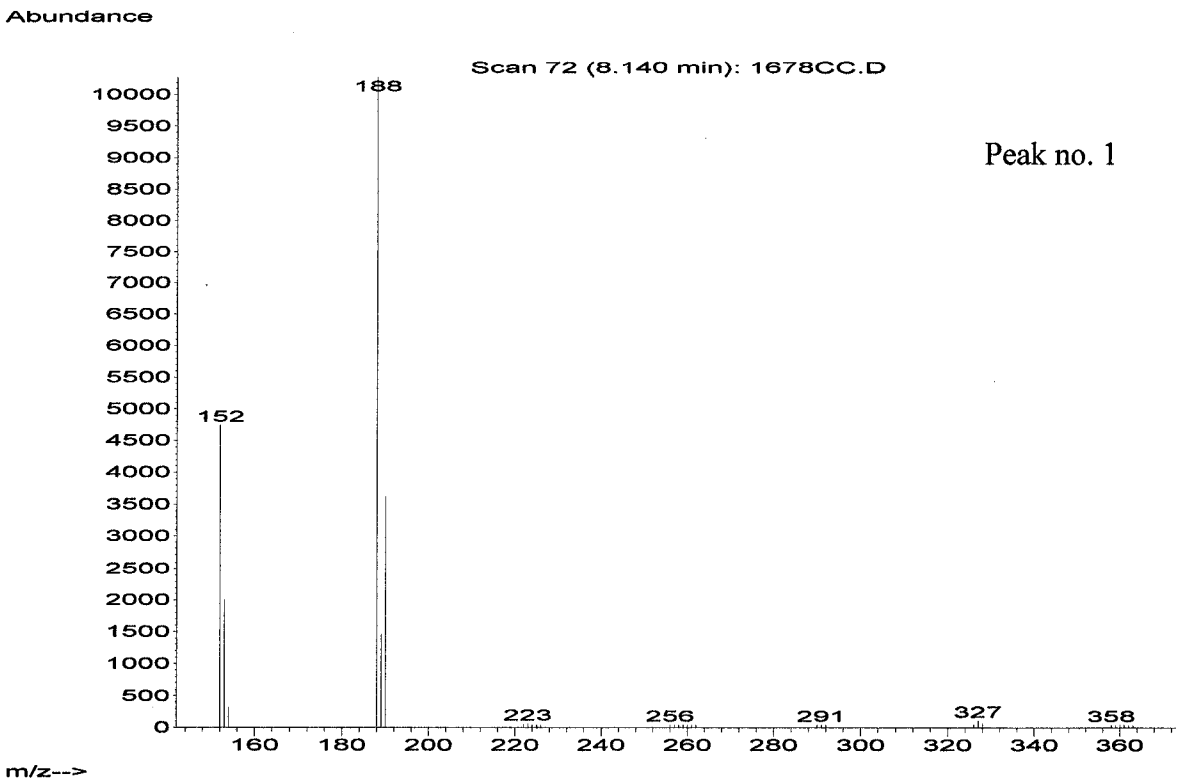
Zwiernik, M.J., Quensen III, J.F., and Boyd, S.A. (1998). $FeSO_4$ amendments stimulate extensive anaerobic PCB dechlorination. Environ. Sci. Technol. **32**: 3360-3365.

Appendix A

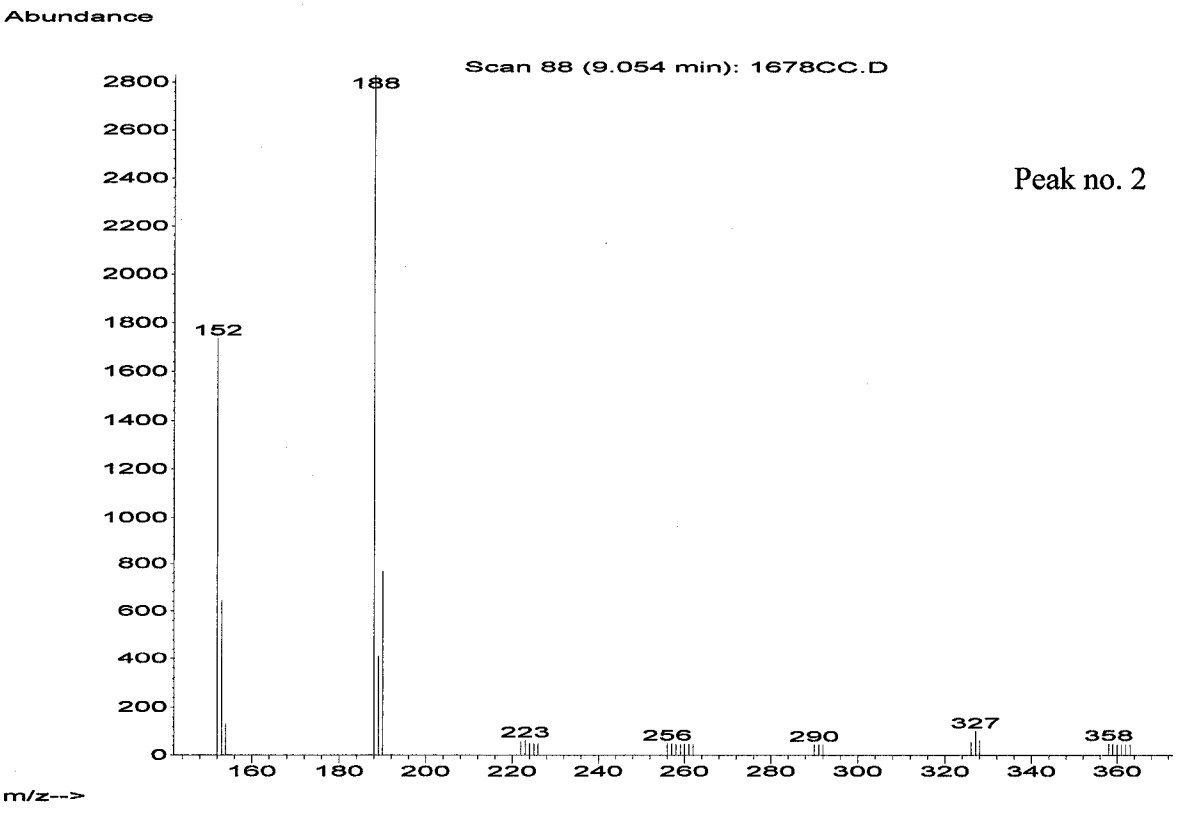
Gas chromatogram and mass spectra of polychlorinated biphenyl congeners present in Aroclor 1232



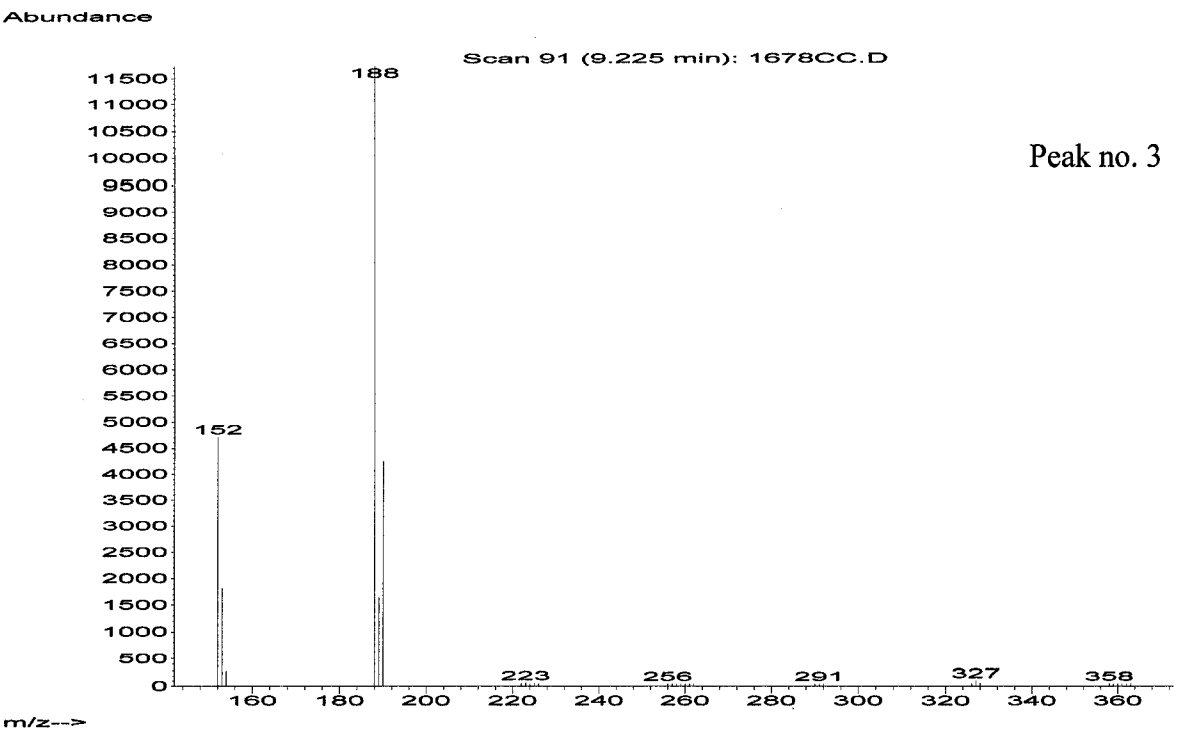
Representative chromatogram of extracts containing Aroclor 1232 showing peaks corresponding to mono- to hexachlorobiphenyls (See mass spectra of identified peaks below). The identity of each peak is shown in Table 3.2 in Chapter 3.



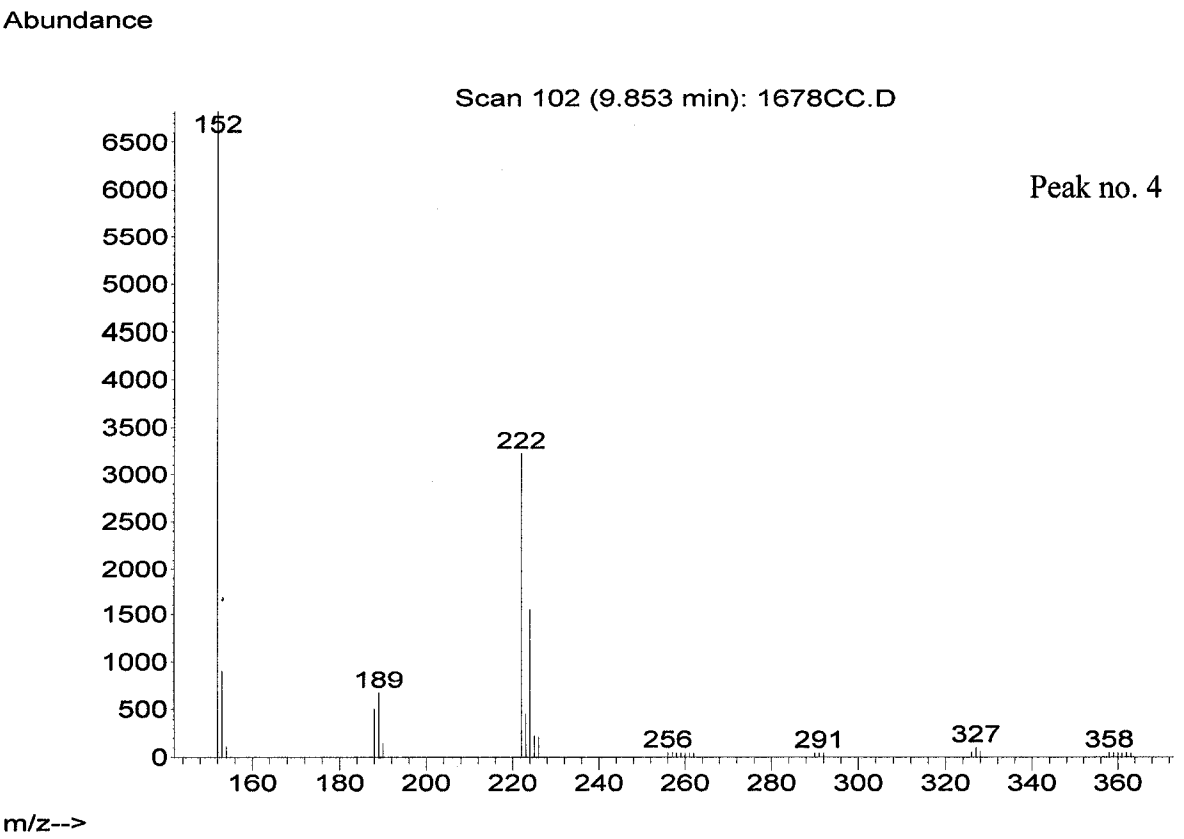
Peak no. 1



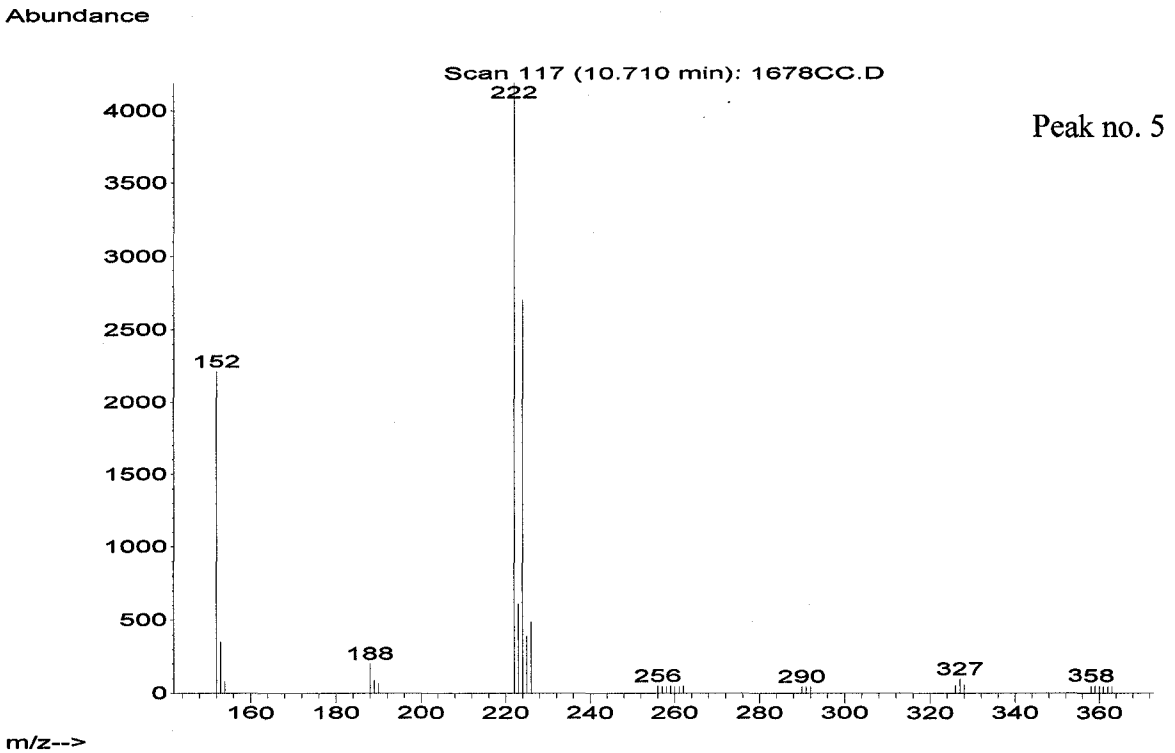
Peak no. 2



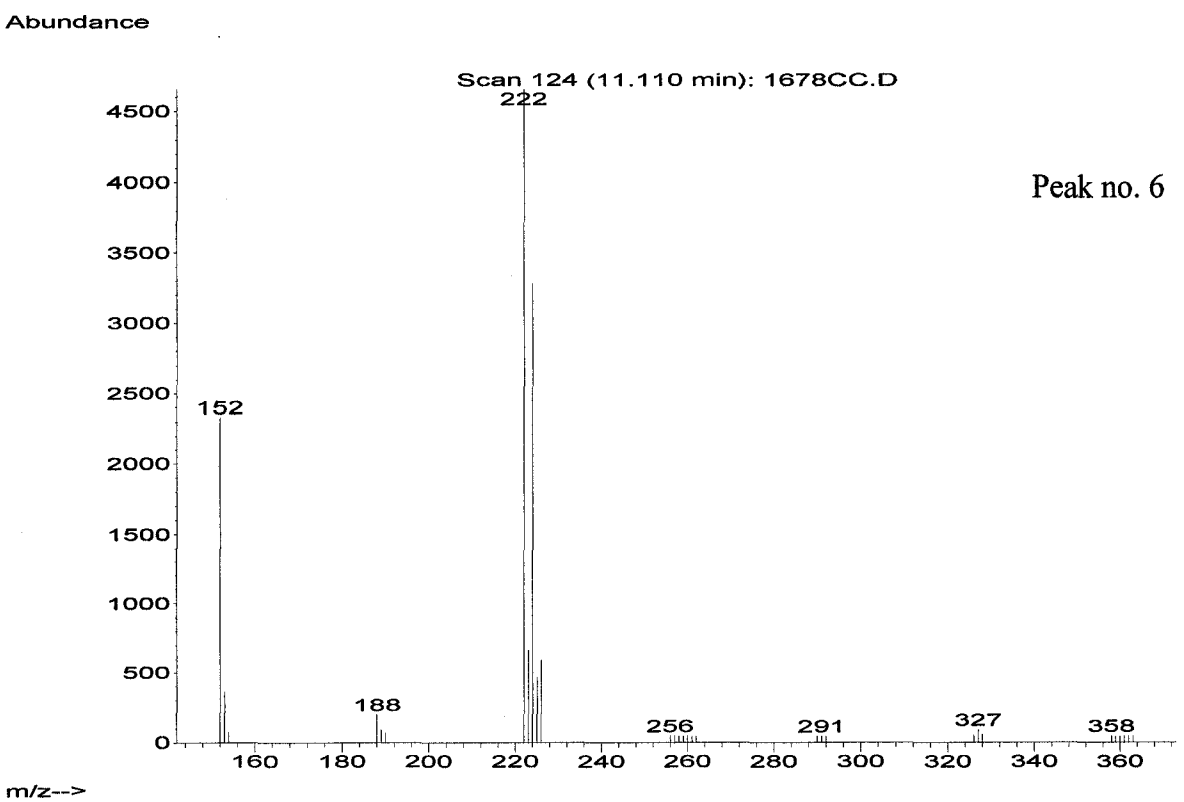
Peak no. 3



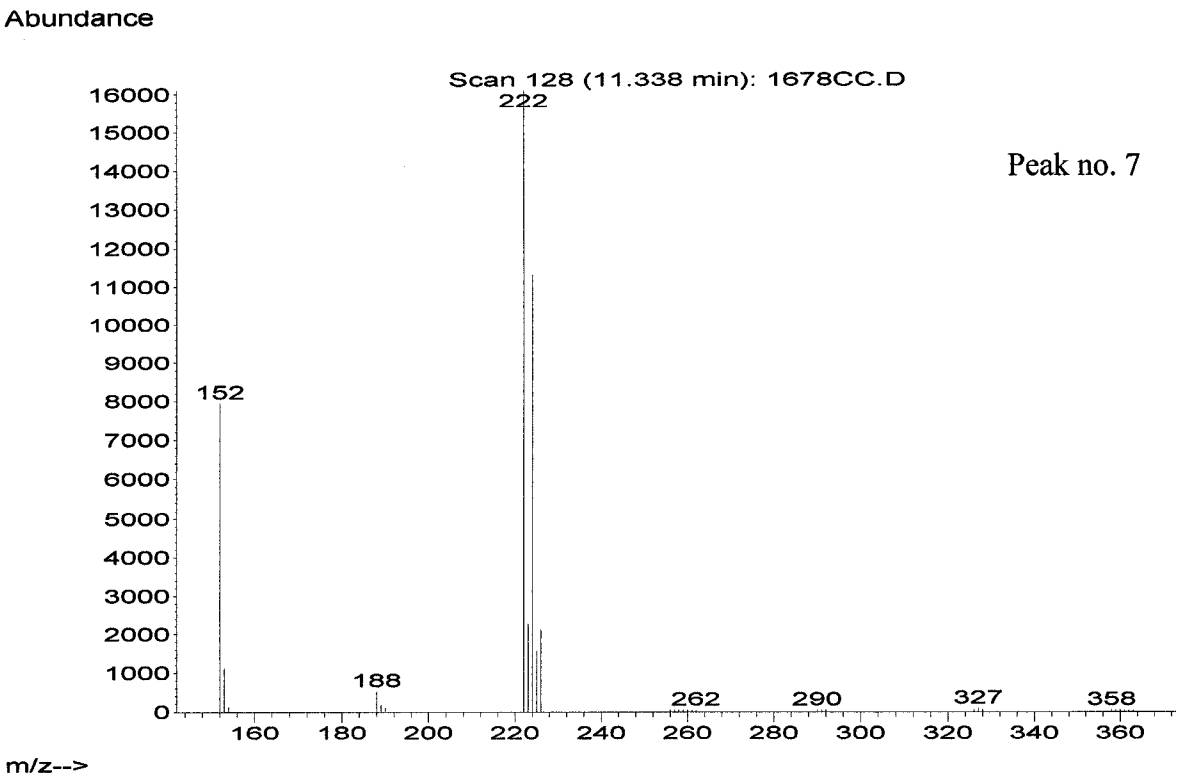
Peak no. 4

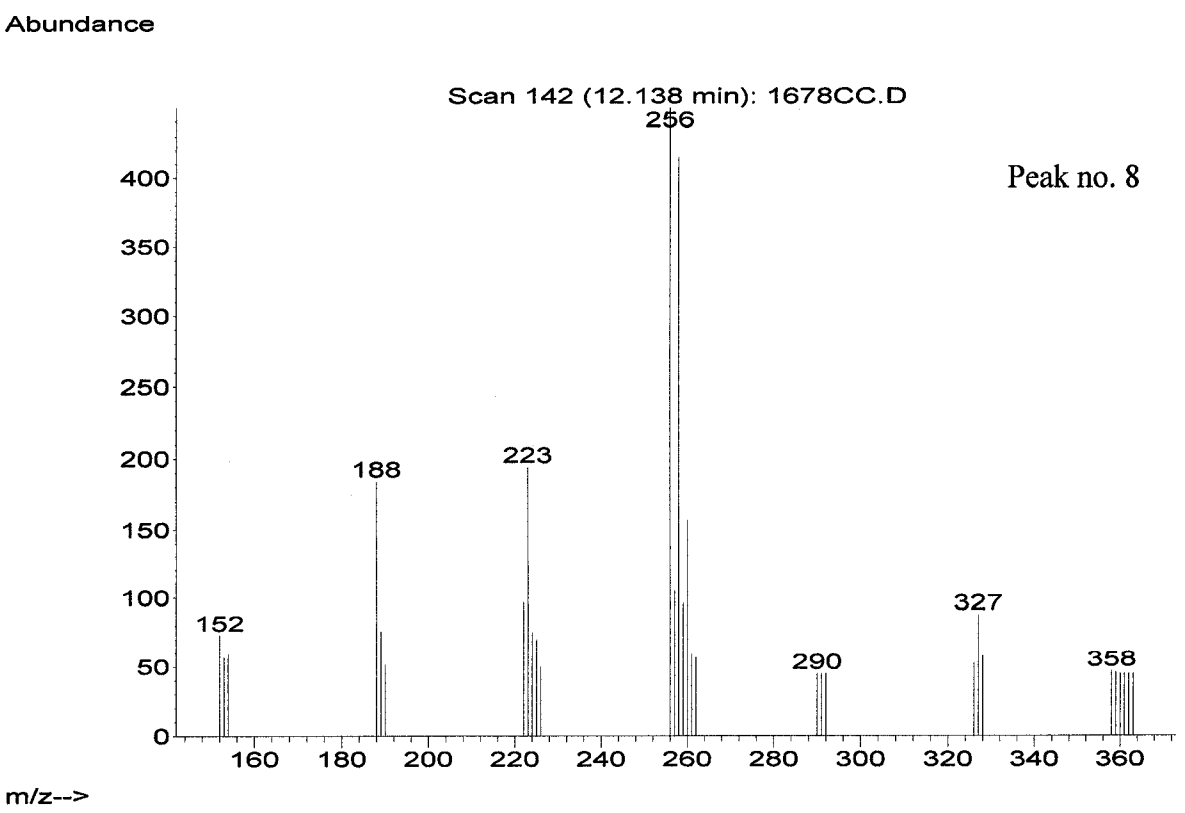


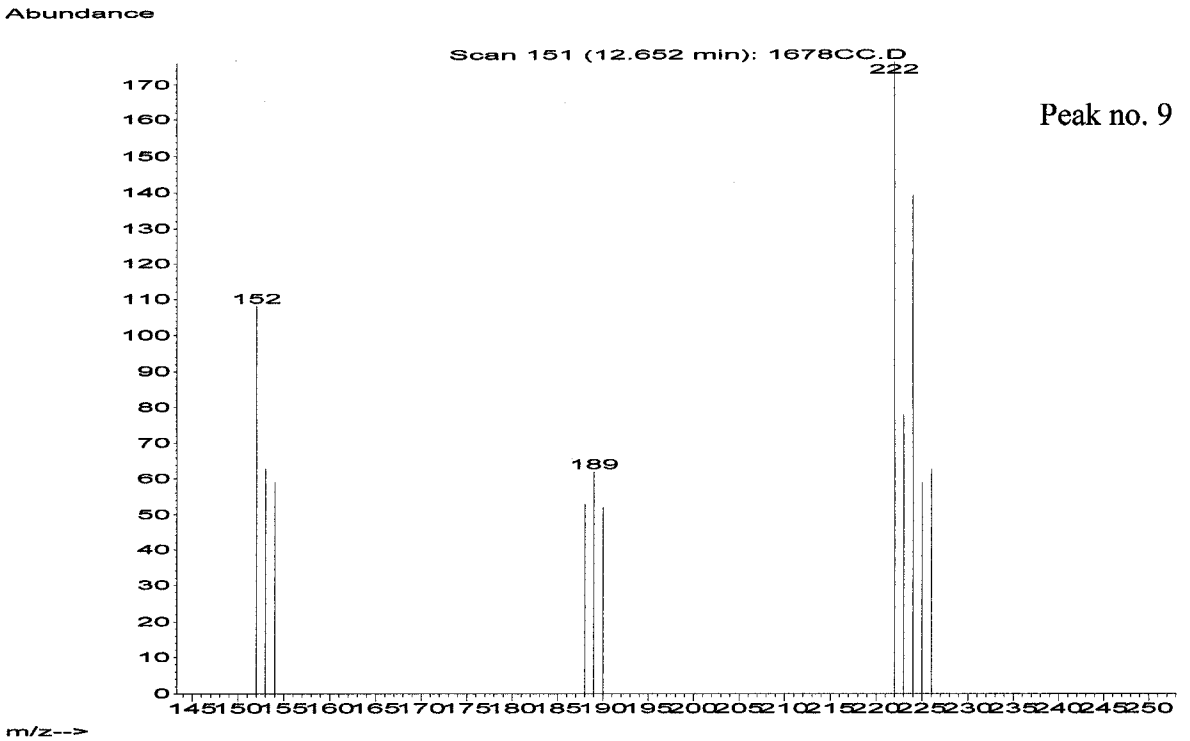
Peak no. 5



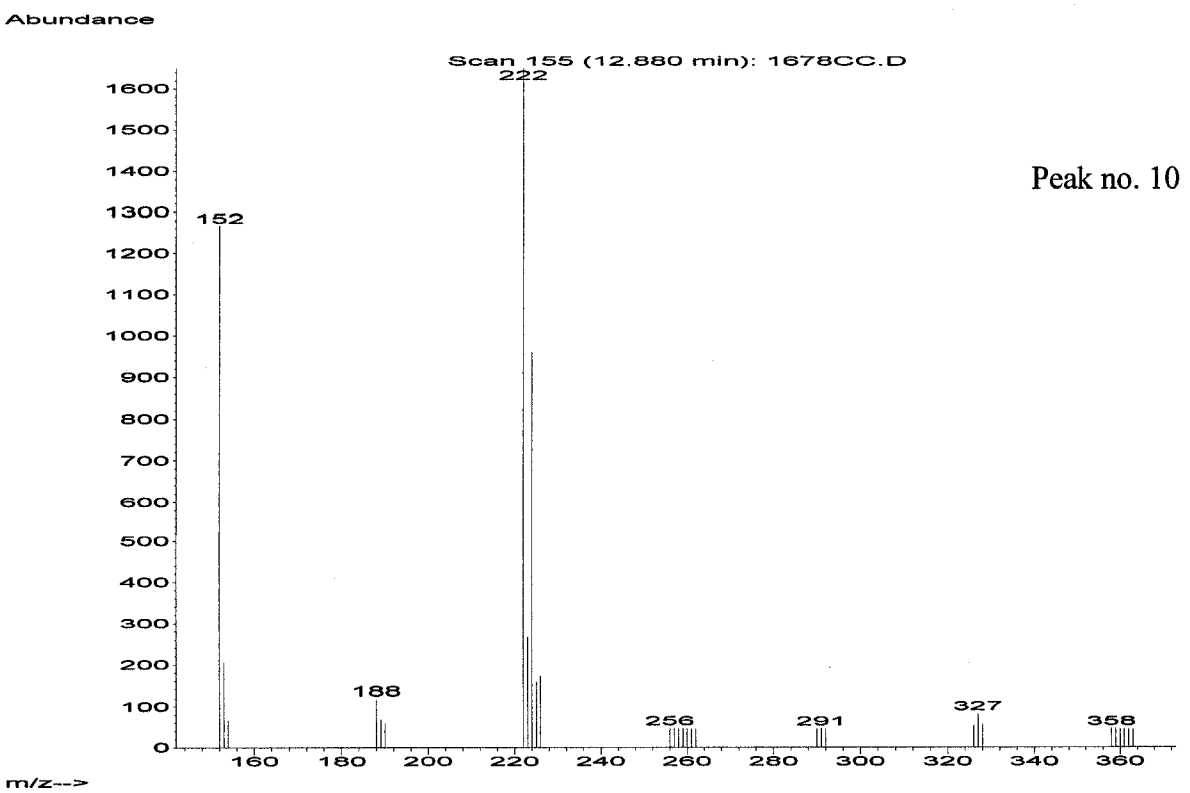
Peak no. 6


Abundance
Scan 128 (11.338 min): 1678CC.D
222
Peak no. 7
16000
15000
14000
13000
12000
11000
10000
9000
8000
7000
6000
5000
4000
3000
2000
1000
0
152
188
262
290
327
358
160
180
200
220
240
260
280
300
320
340
360
m/z-->

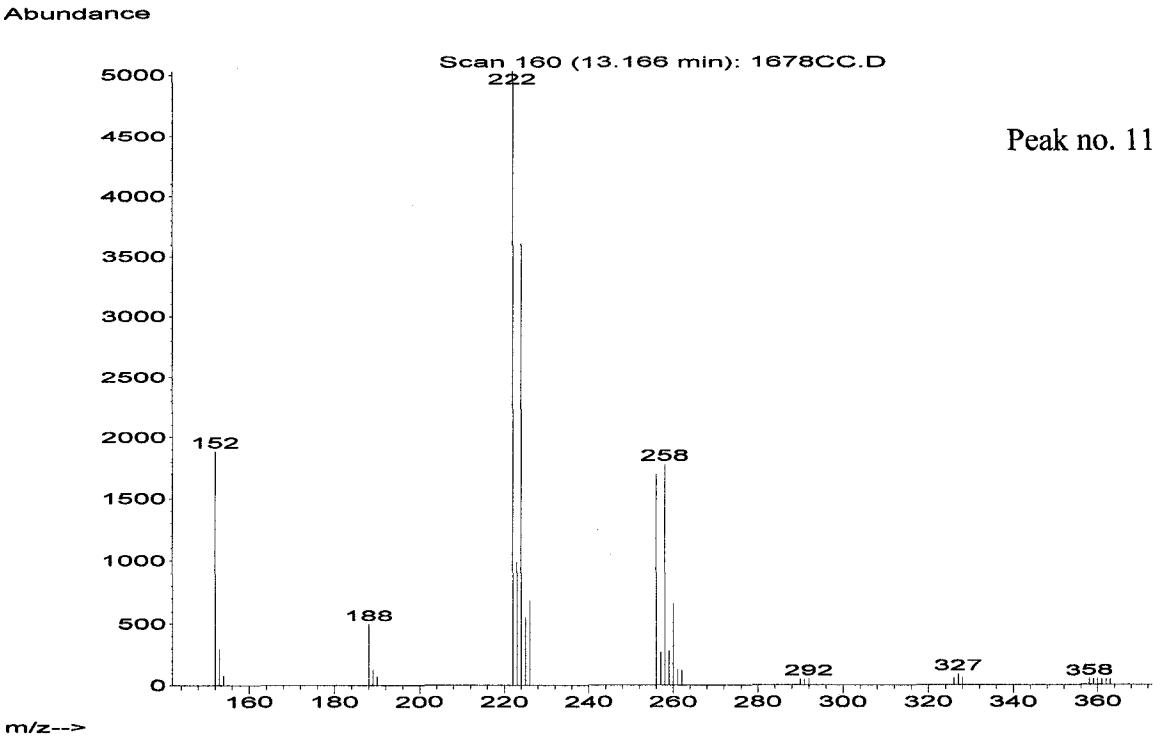

Abundance
Scan 142 (12.138 min): 1678CC.D
256
Peak no. 8
400
350
300
250
200
150
100
50
0
188
223
152
327
290
358
160
180
200
220
240
260
280
300
320
340
360
m/z-->



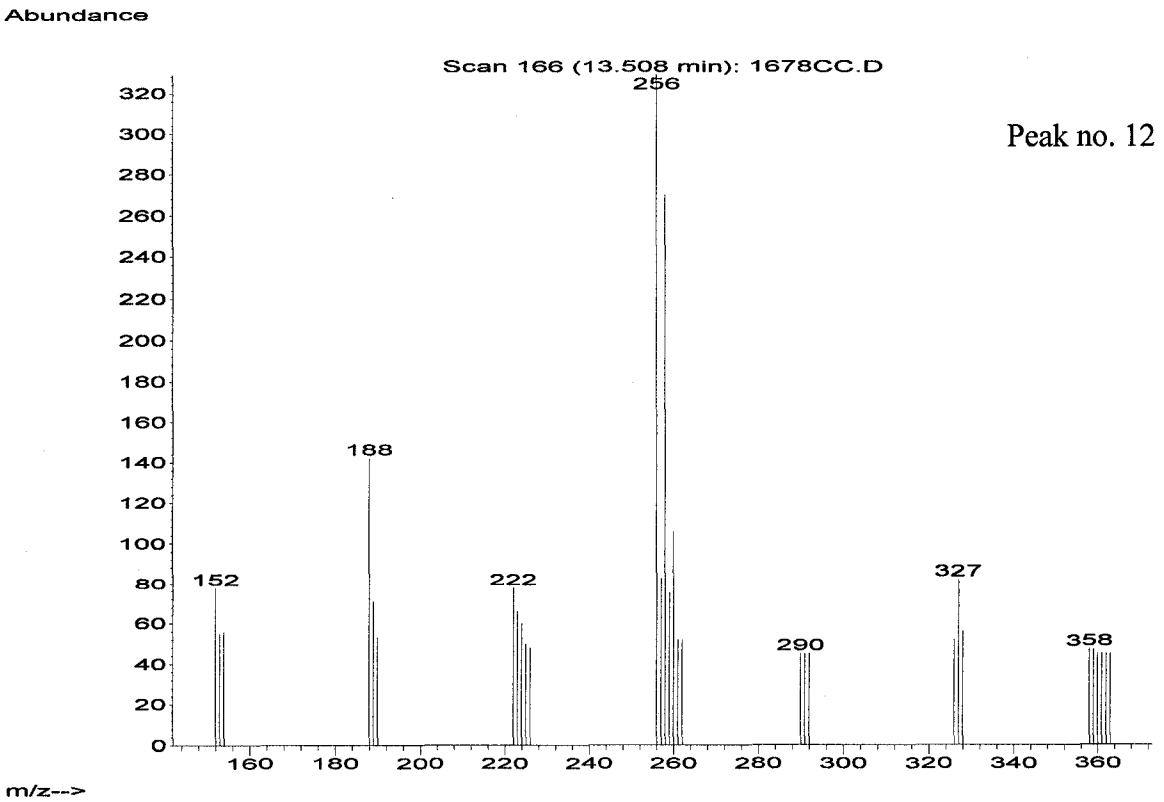
Peak no. 9



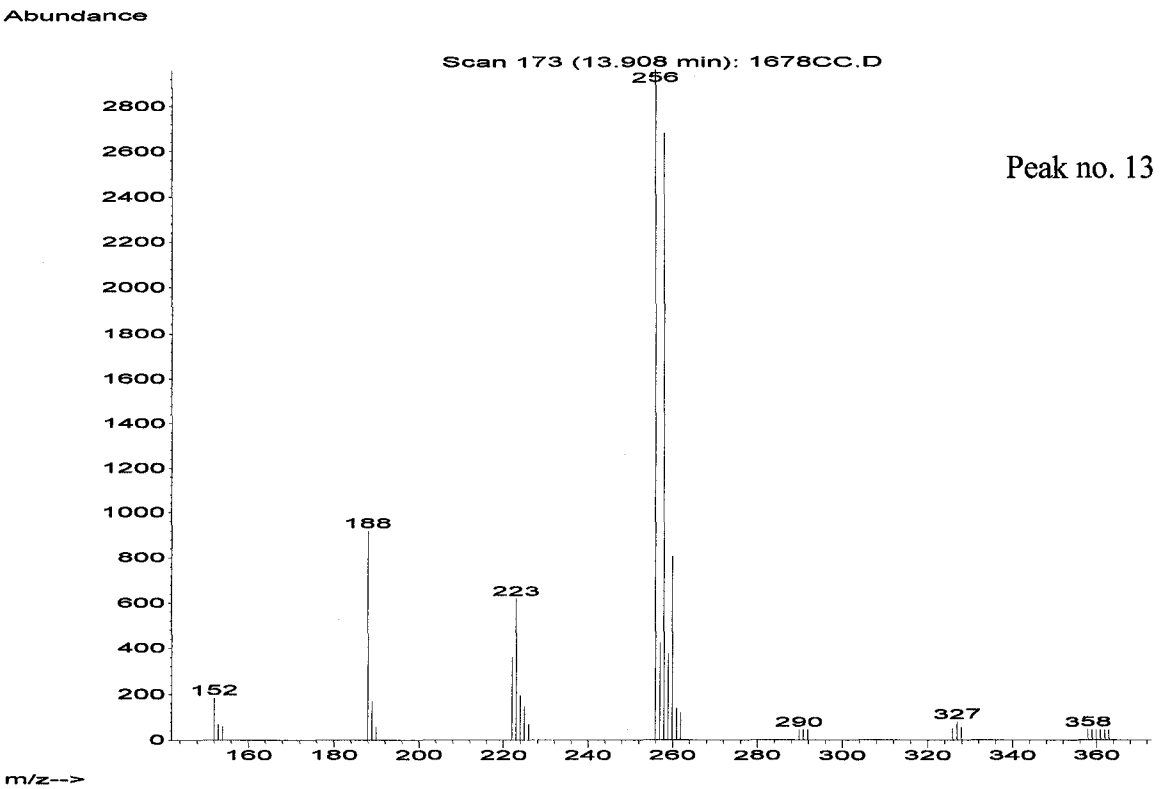
Peak no. 10



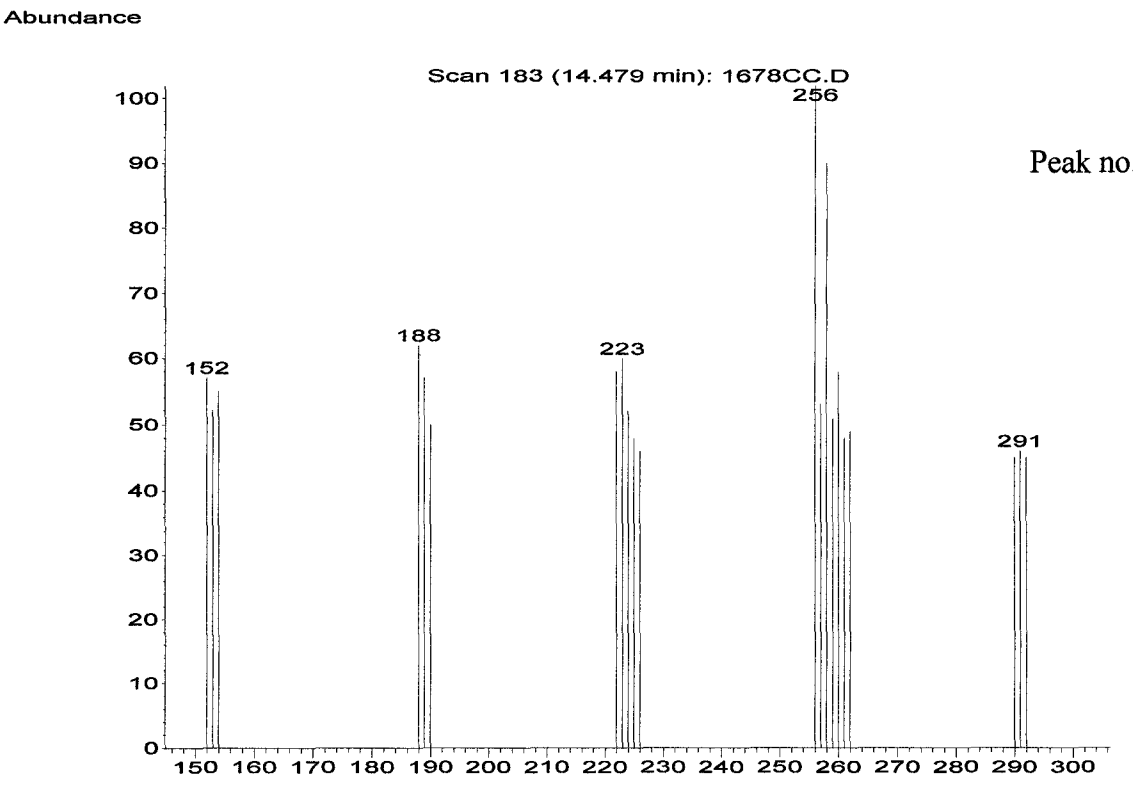
Peak no. 11



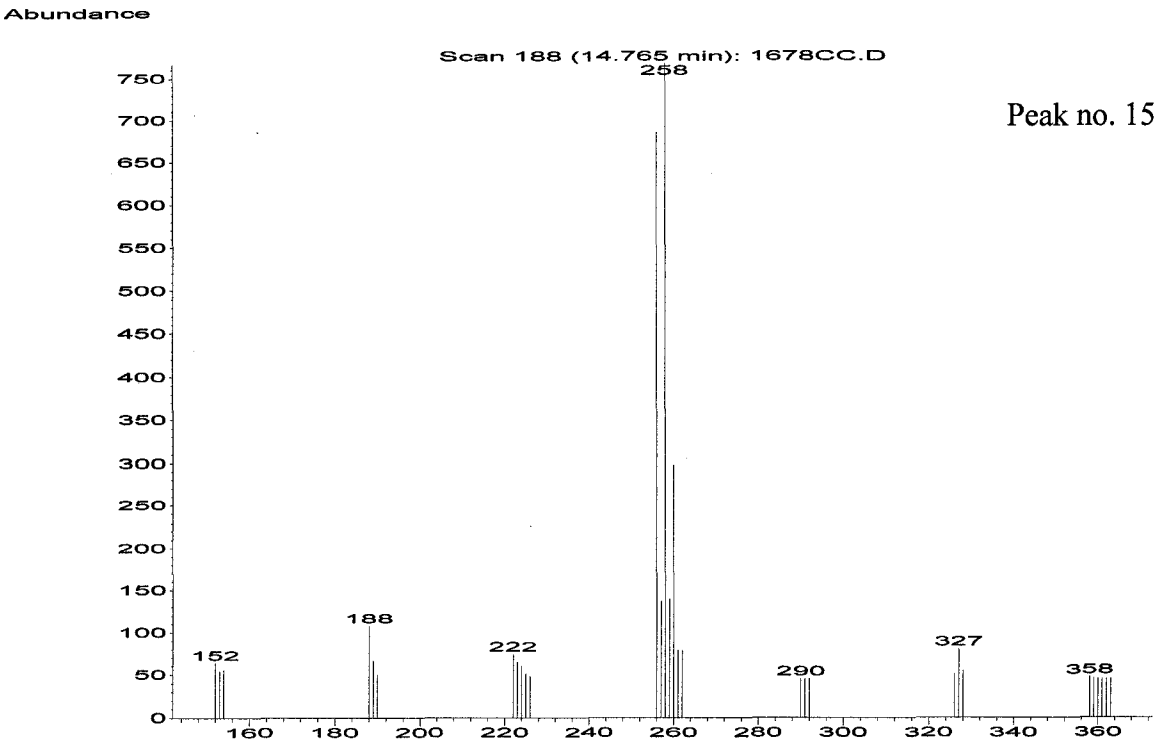
Peak no. 12

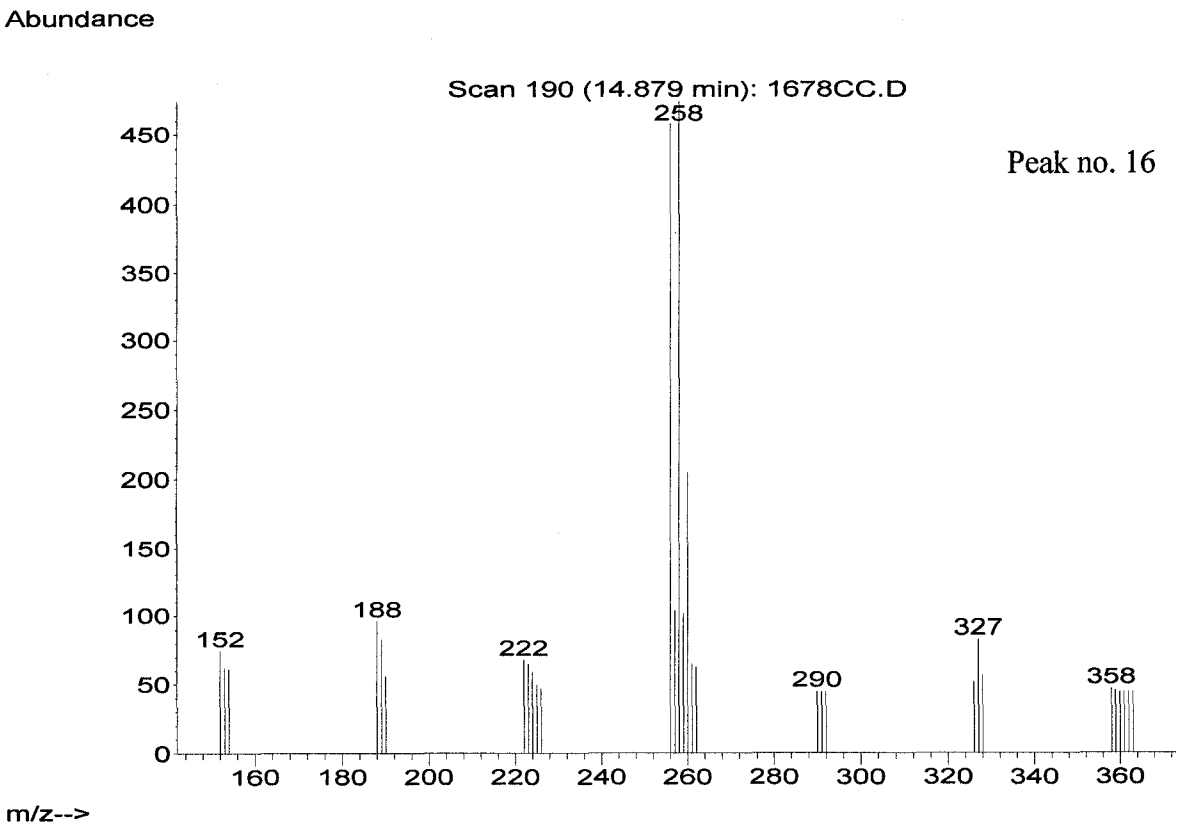


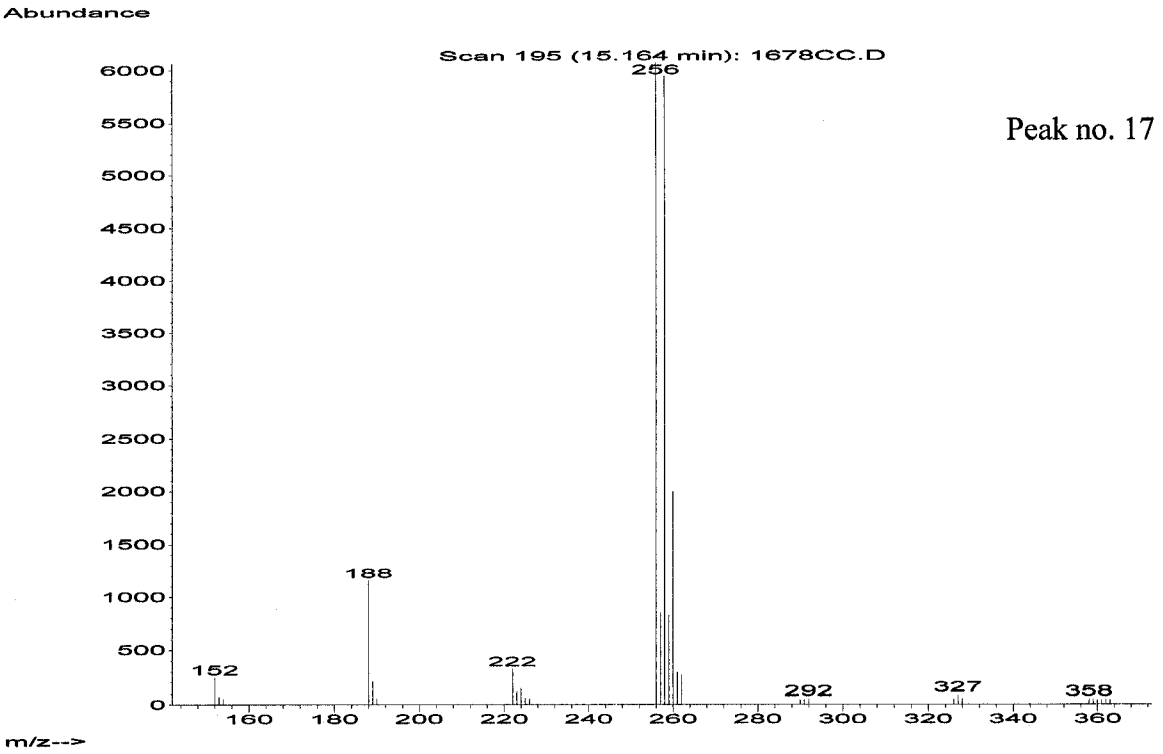
Peak no. 13



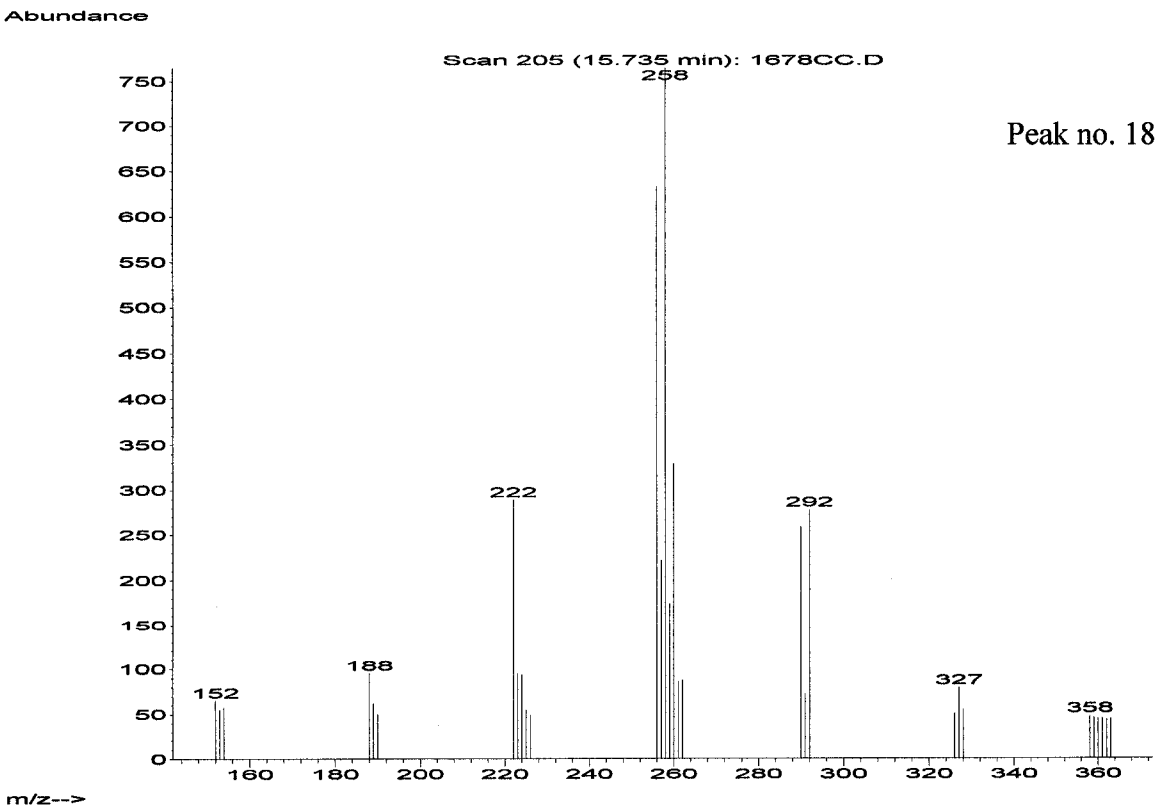
Peak no. 14


Abundance
Scan 188 (14.765 min): 1678CC.D
258
Peak no. 15
750
700
650
600
550
500
450
400
350
300
250
200
150
100
50
0
152
188
222
290
327
358
160
180
200
220
240
260
280
300
320
340
360
m/z-->

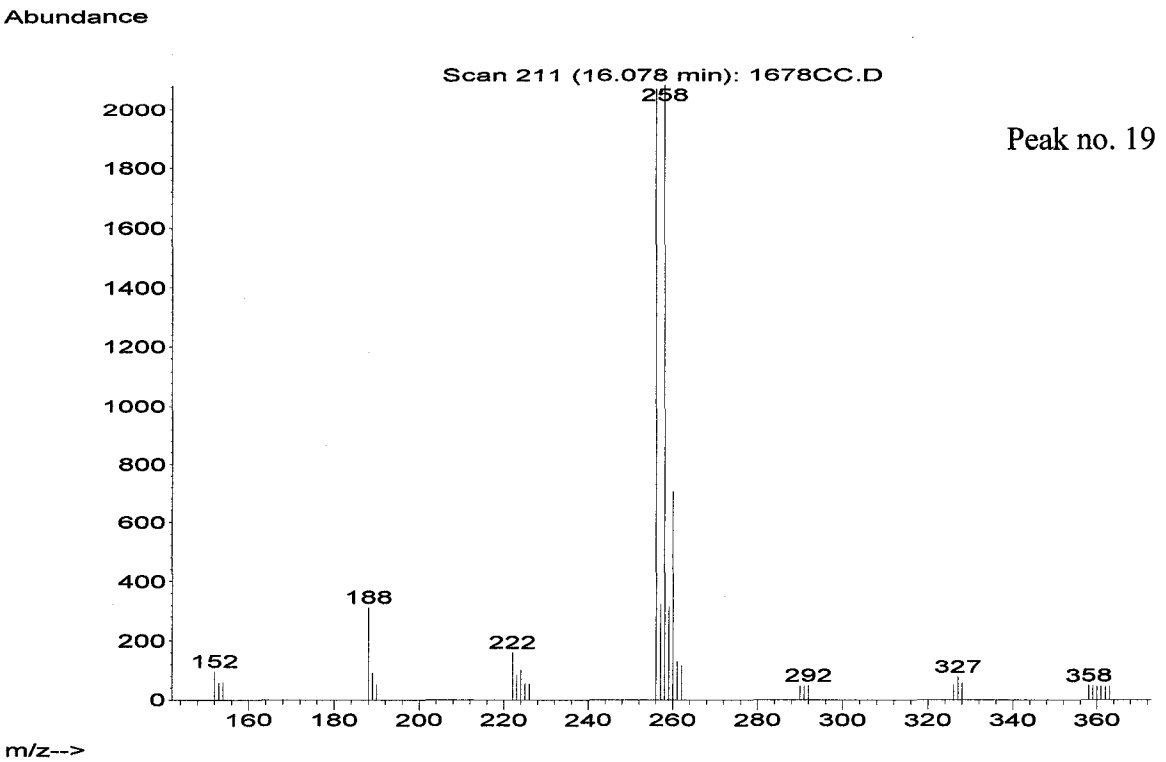

Abundance
Scan 190 (14.879 min): 1678CC.D
258
Peak no. 16
450
400
350
300
250
200
150
100
50
0
152
188
222
290
327
358
160
180
200
220
240
260
280
300
320
340
360
m/z-->



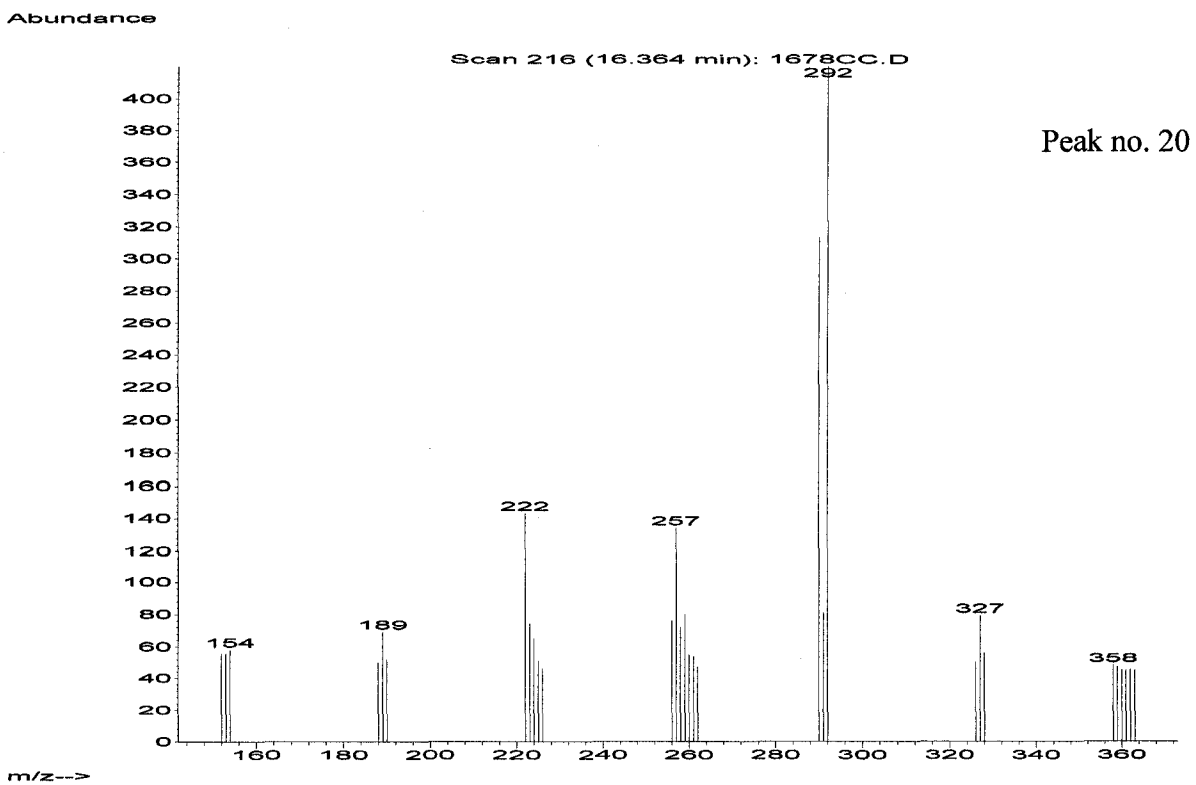
Peak no. 17



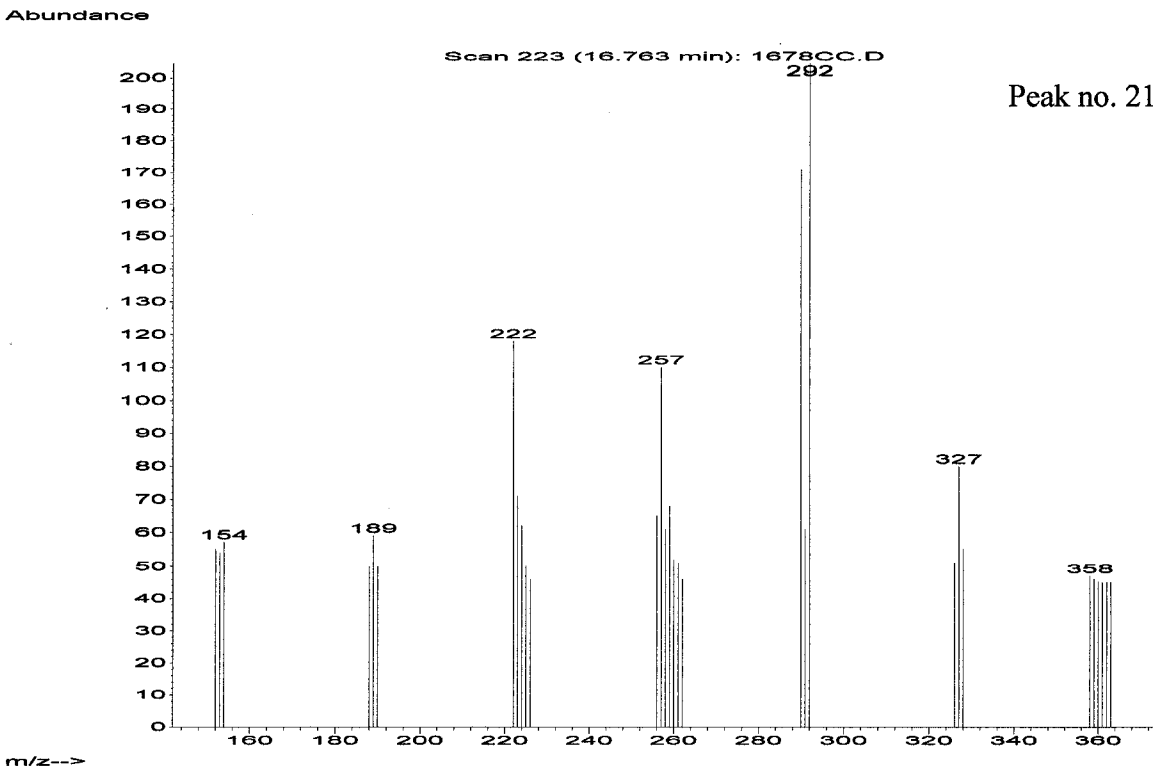
Peak no. 18

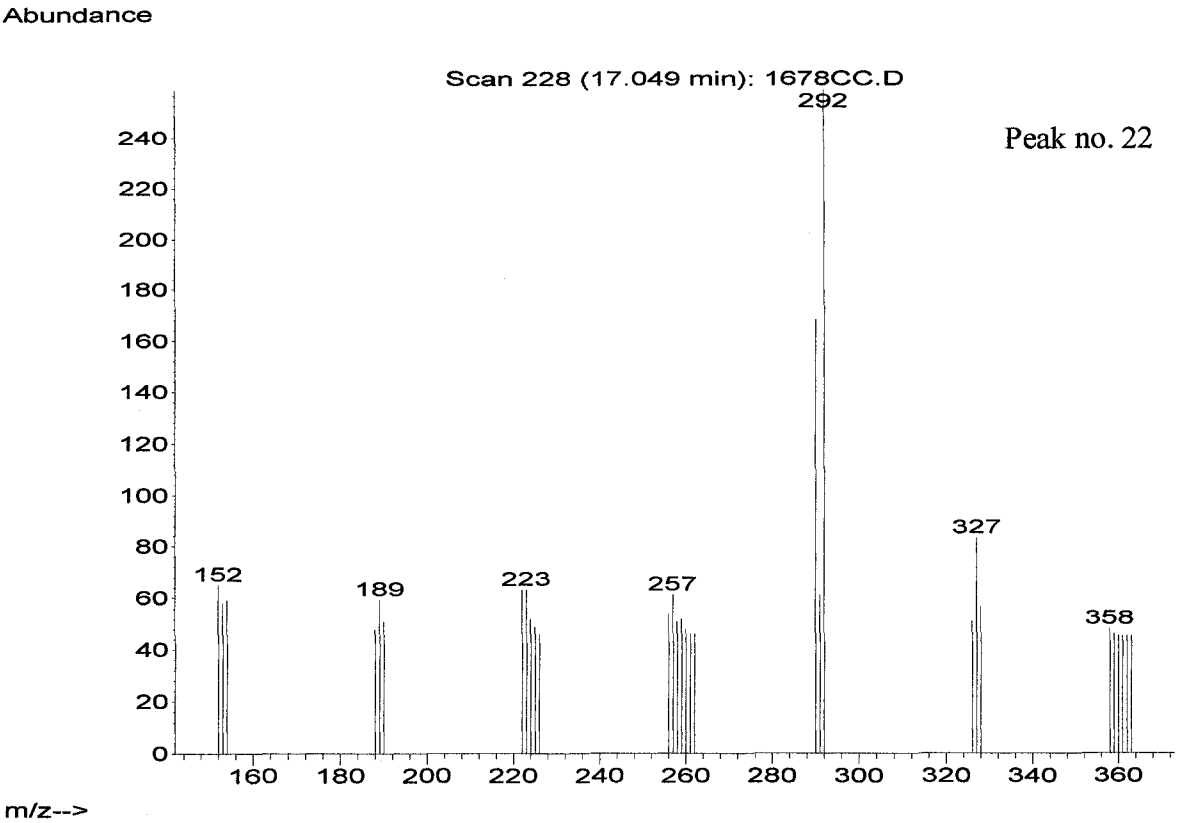


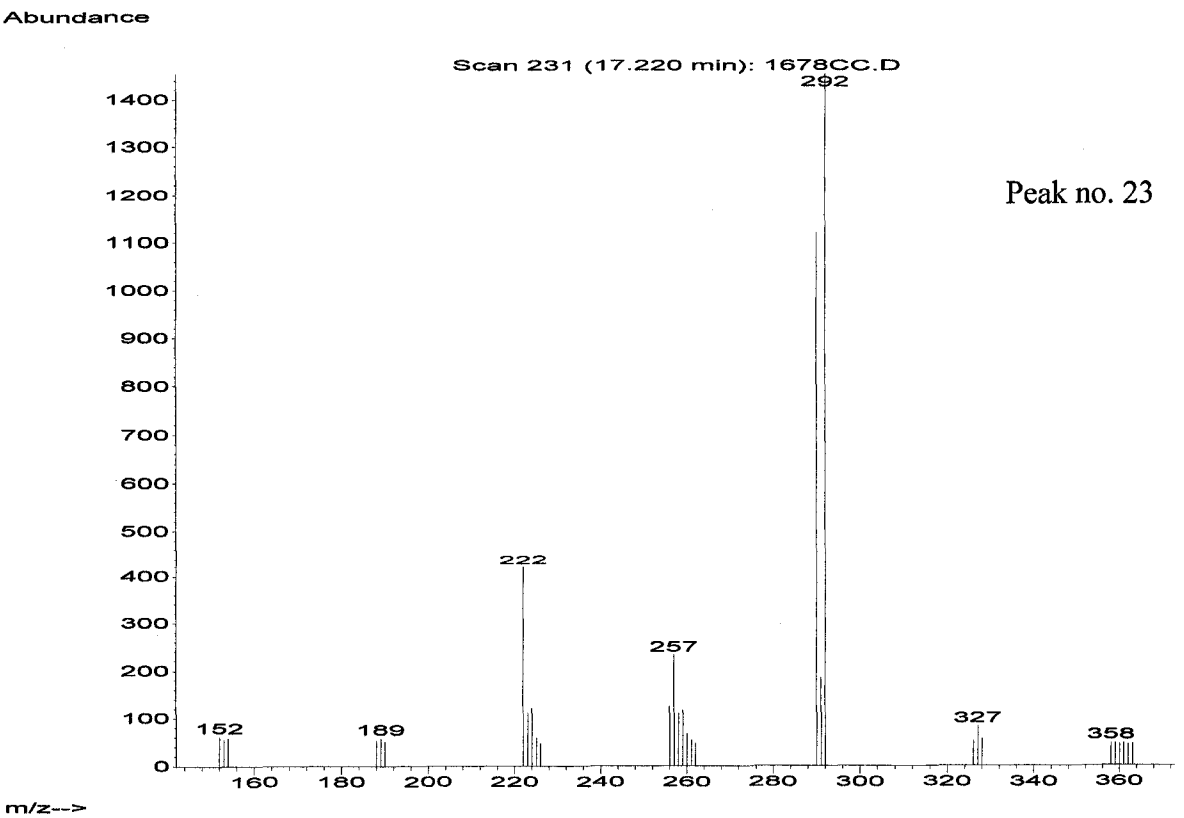
Peak no. 19

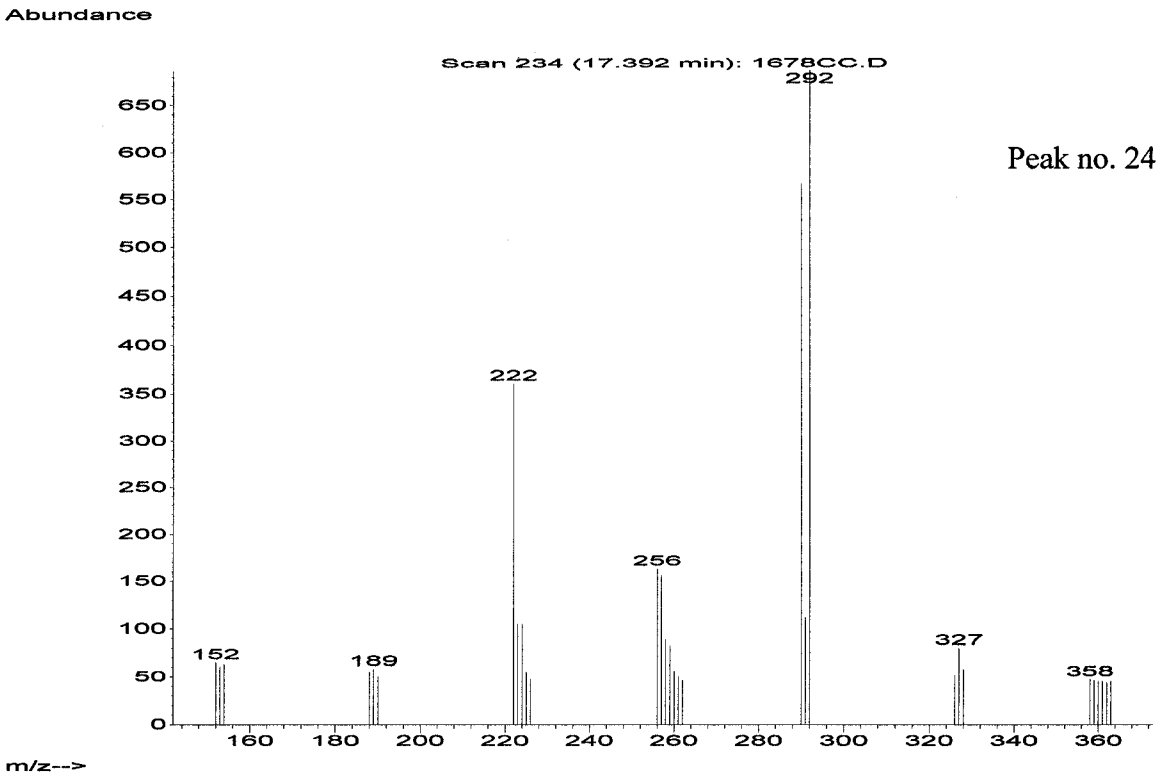


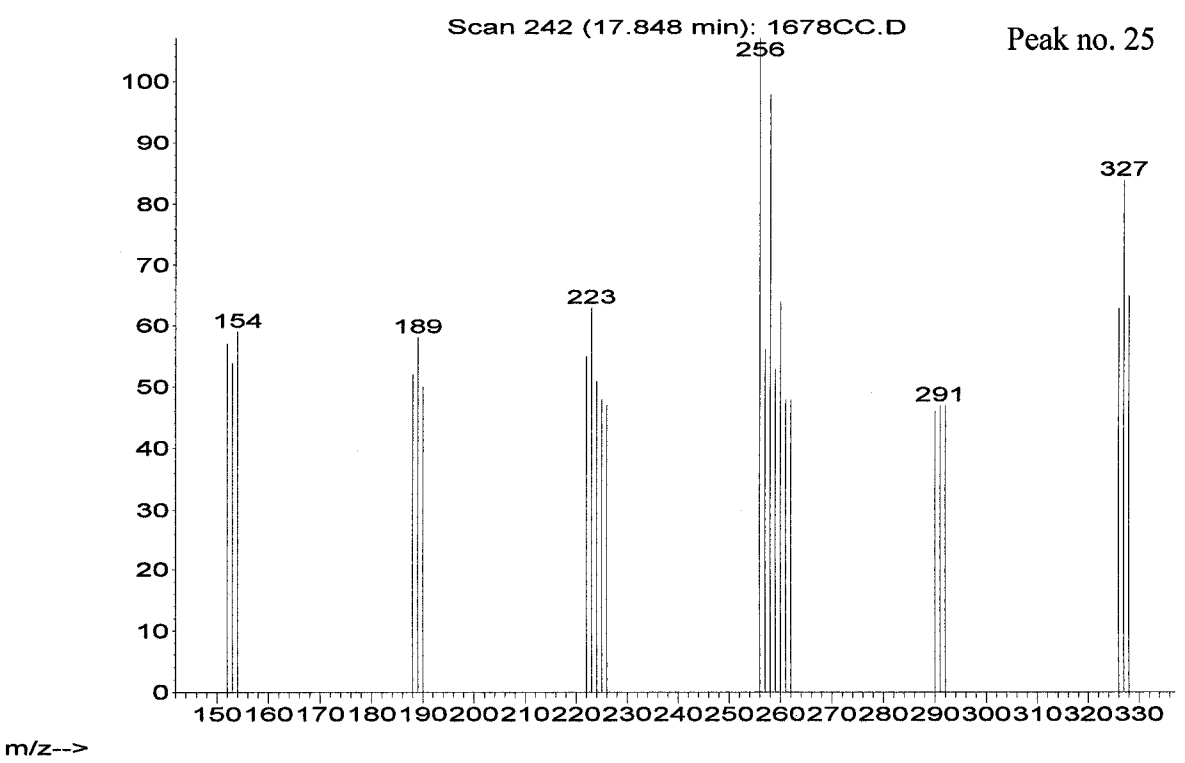
Peak no. 20

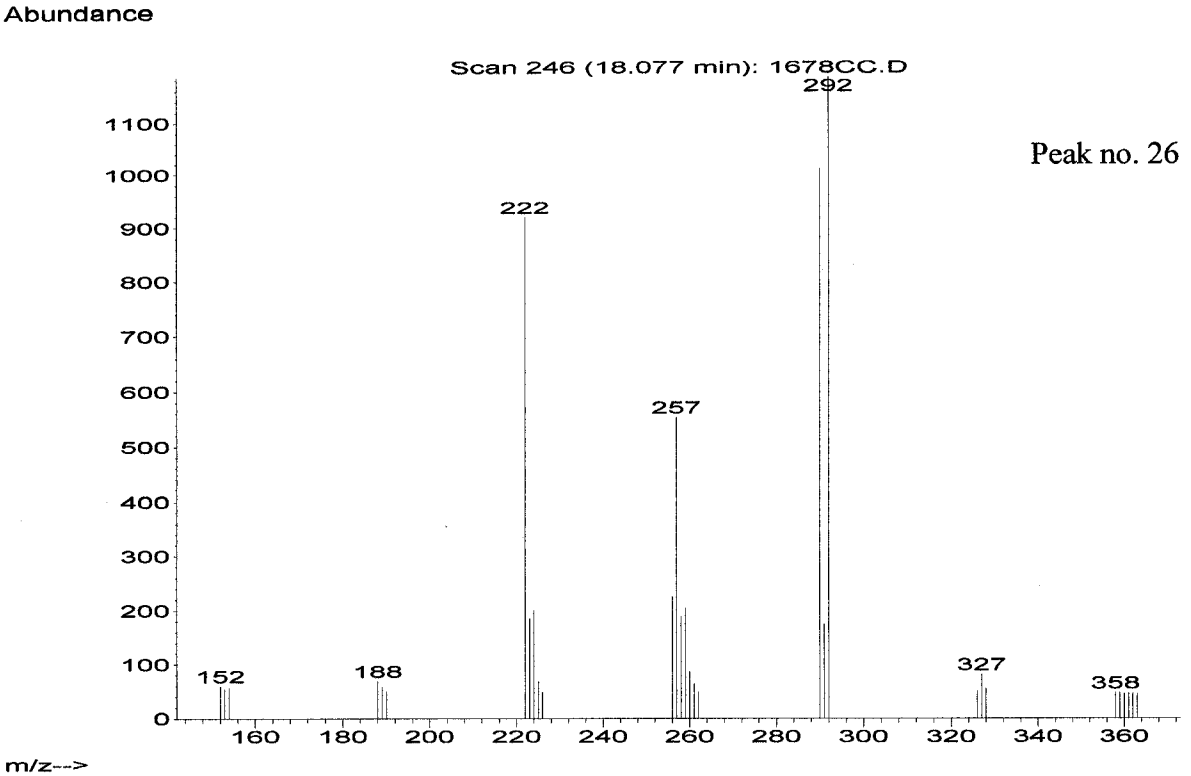

Abundance
Scan 223 (16.763 min): 1678CC.D
292
Peak no. 21
222
257
327
154
189
358
m/z-->


Abundance
Scan 228 (17.049 min): 1678CC.D
292
Peak no. 22
240
220
200
180
160
140
120
100
80
60
40
20
0
152
189
223
257
327
358
160
180
200
220
240
260
280
300
320
340
360
m/z-->

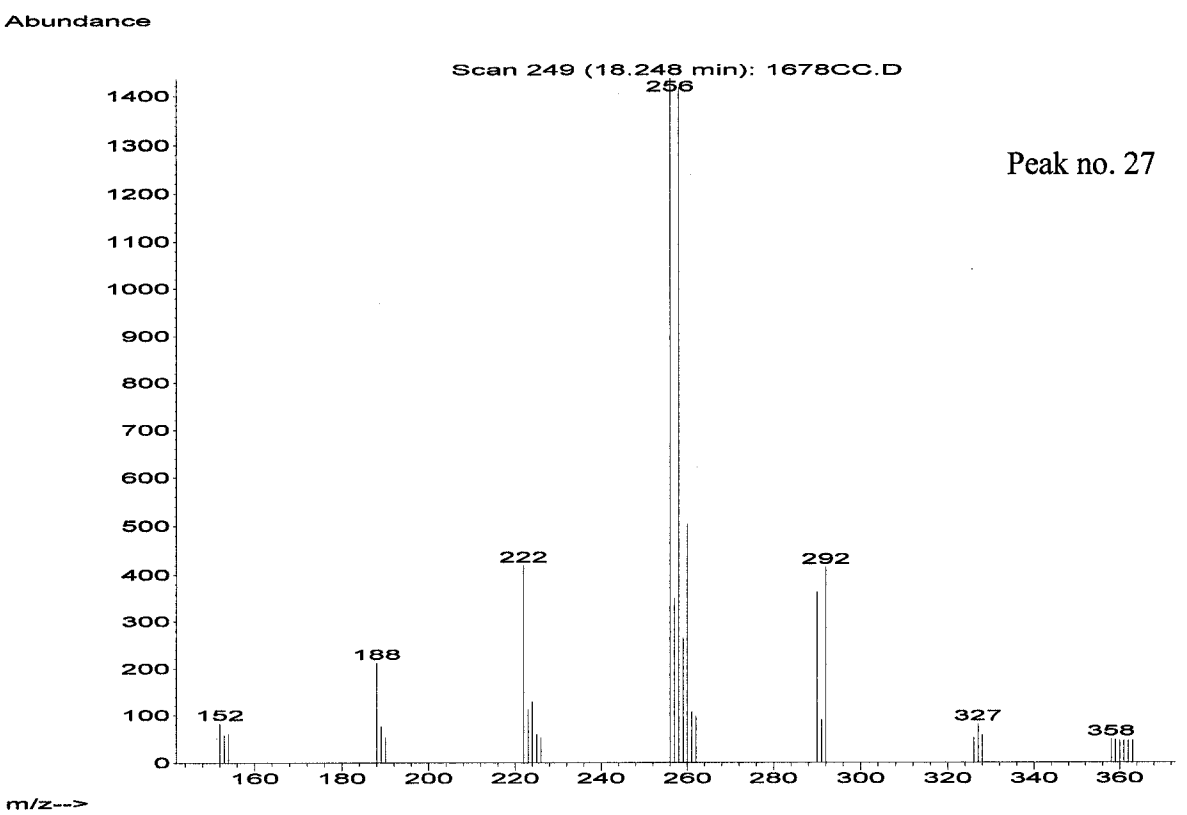

Abundance
Scan 231 (17.220 min): 1678CC.D
292
Peak no. 23
1400
1300
1200
1100
1000
900
800
700
600
500
400
300
200
100
0
222
257
152
189
327
358
160
180
200
220
240
260
280
300
320
340
360
m/z-->


Abundance
Scan 234 (17.392 min): 1678CC.D
292
Peak no. 24
650
600
550
500
450
400
350
300
250
200
150
100
50
0
222
256
152
189
327
358
160
180
200
220
240
260
280
300
320
340
360
m/z-->

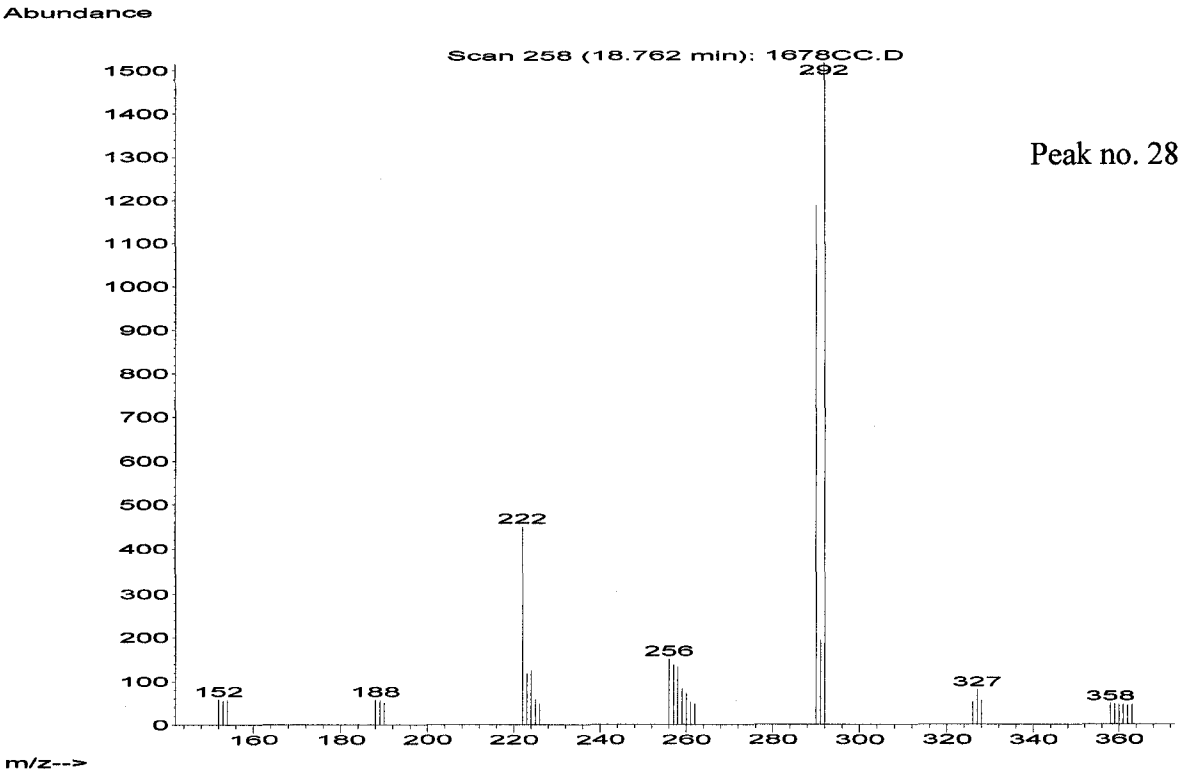

Abundance
Scan 242 (17.848 min): 1678CC.D
256
Peak no. 25
100
90
80
70
60
50
40
30
20
10
0
327
223
154
189
291
150160170180190200210220230240250260270280290300310320330
m/z-->



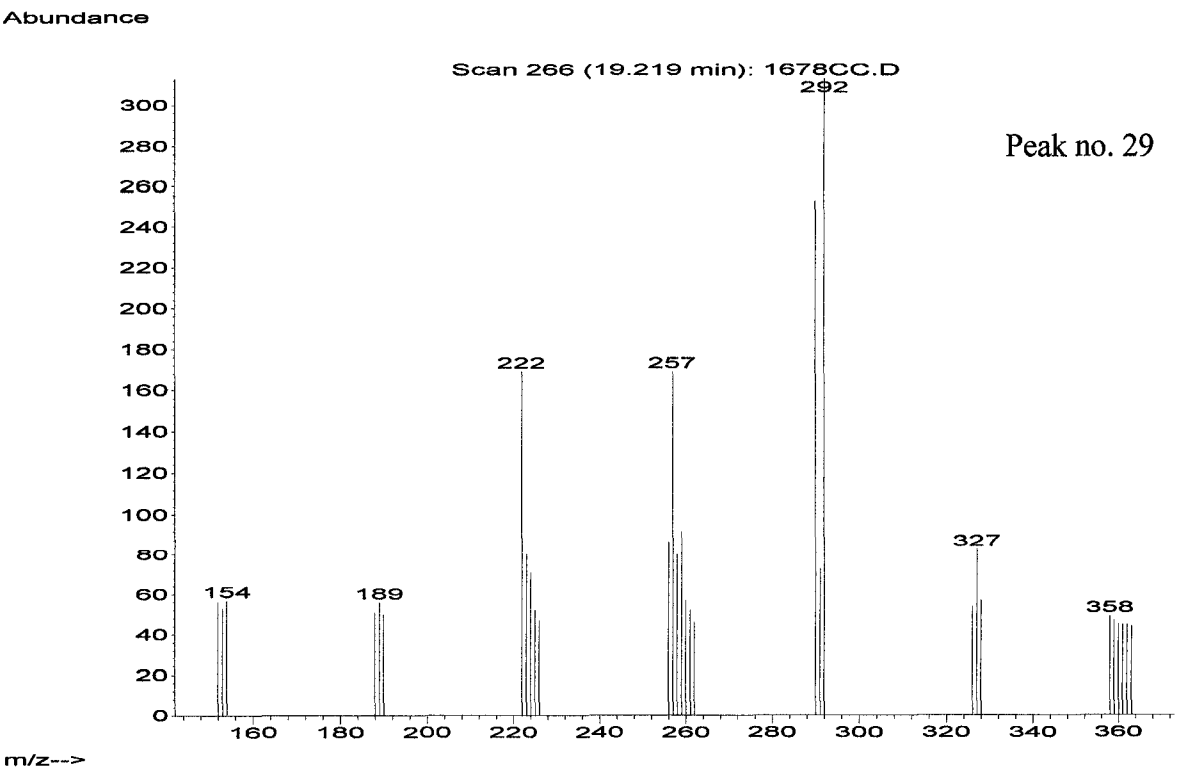
Peak no. 26



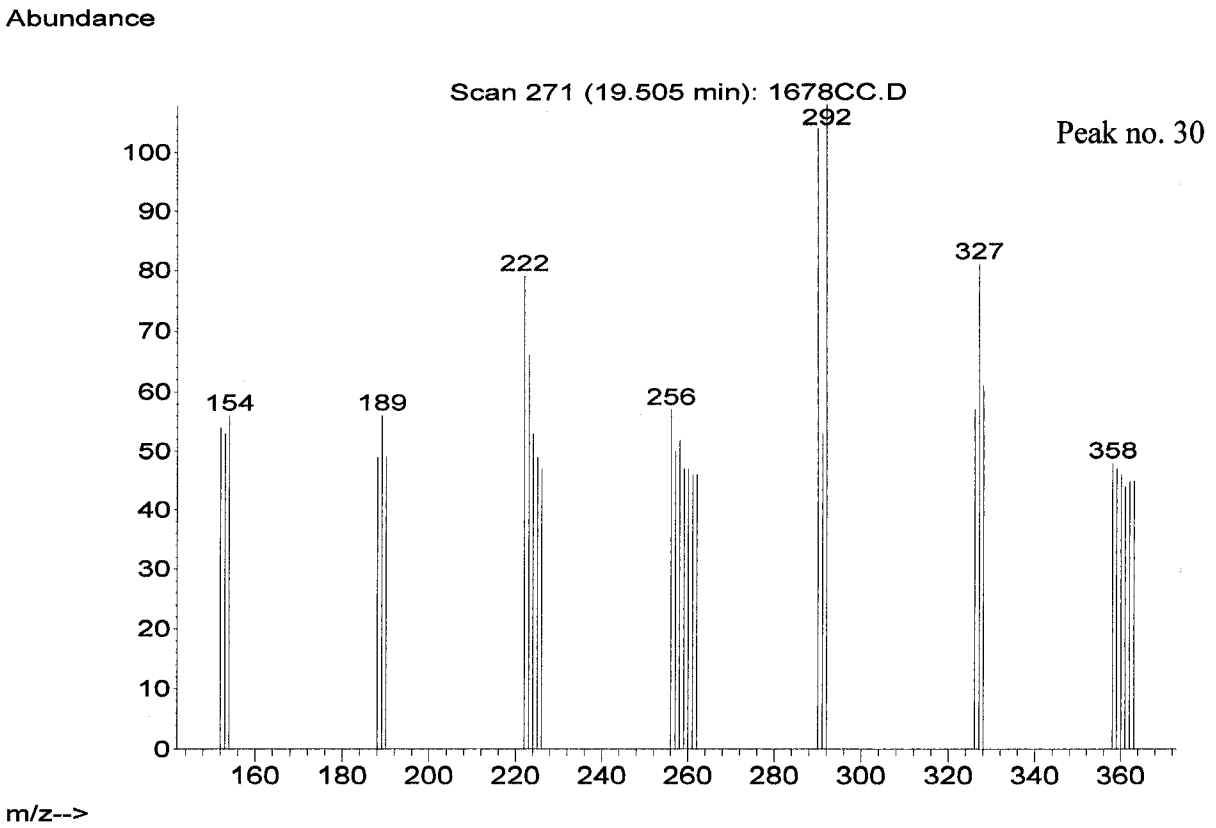
Peak no. 27

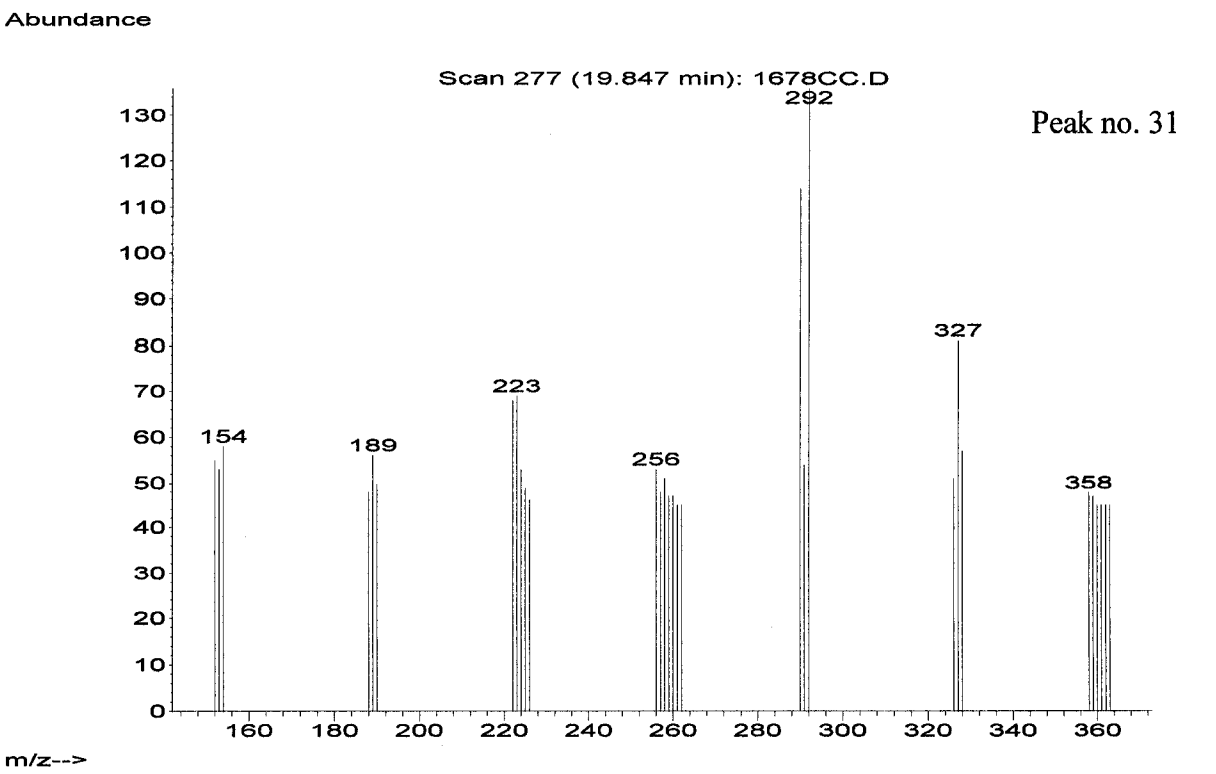


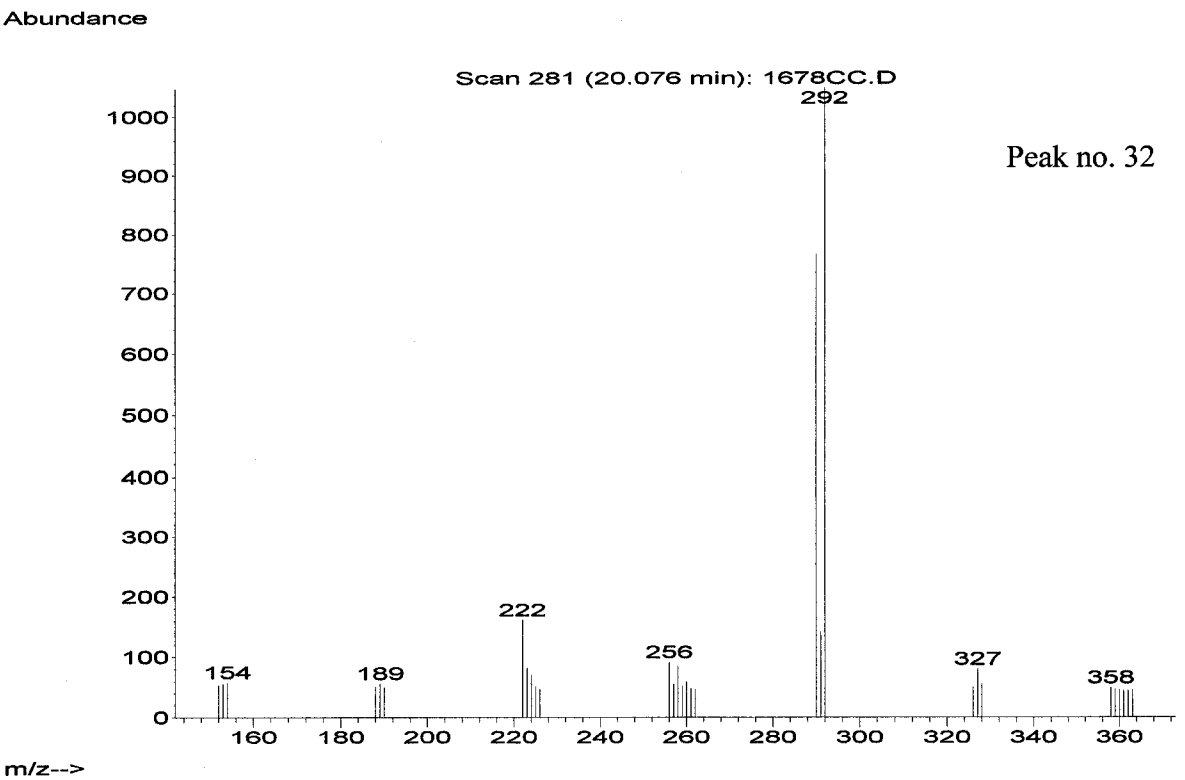
Peak no. 28

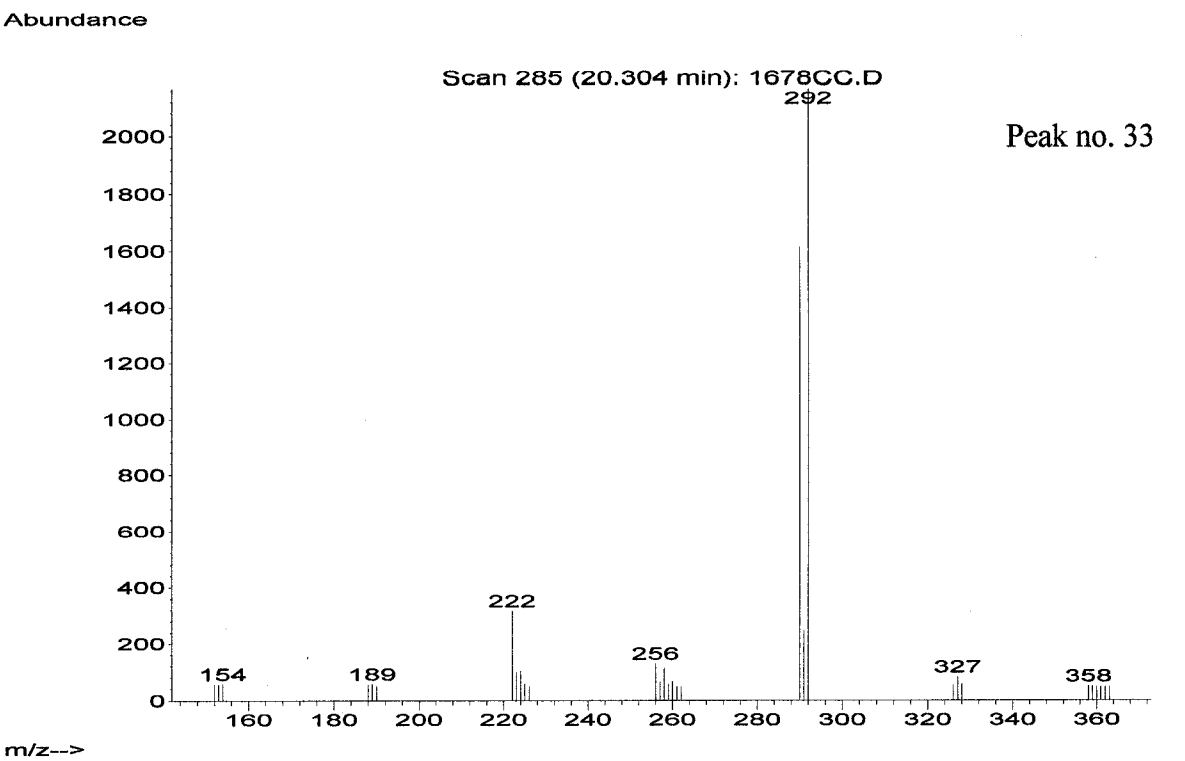


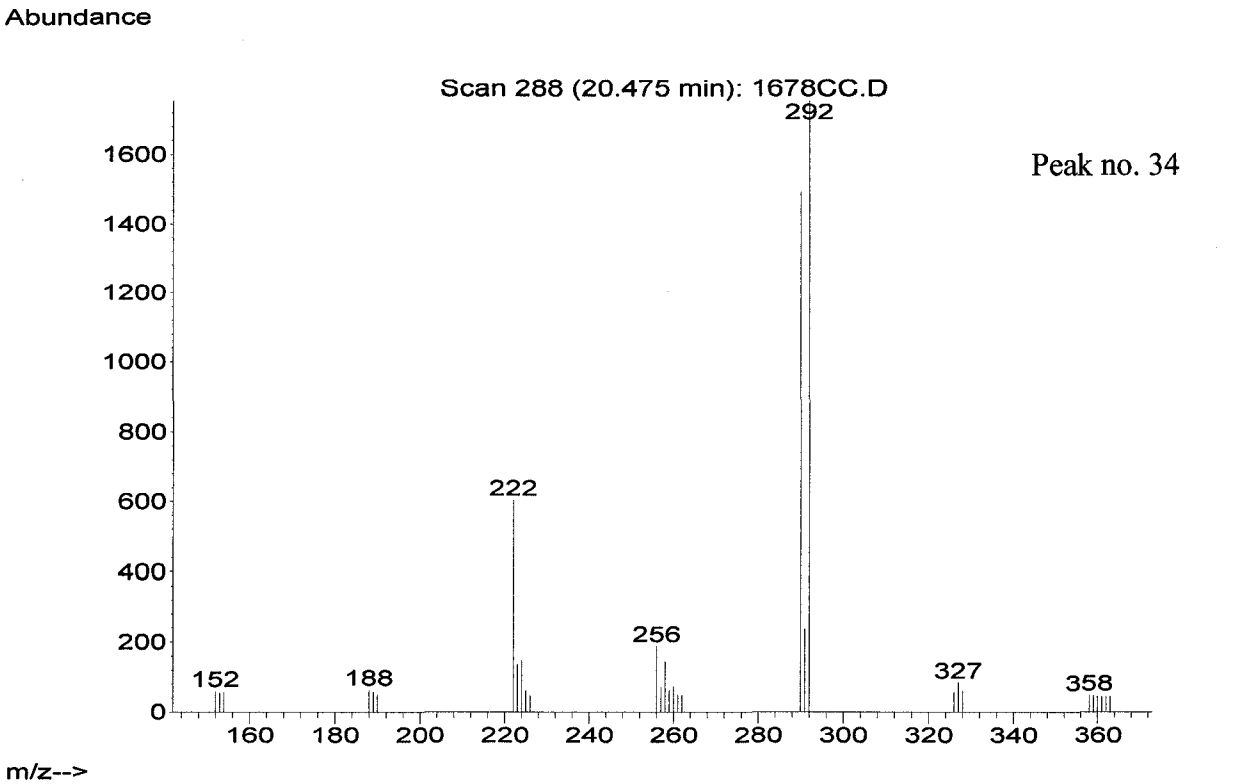
Peak no. 29

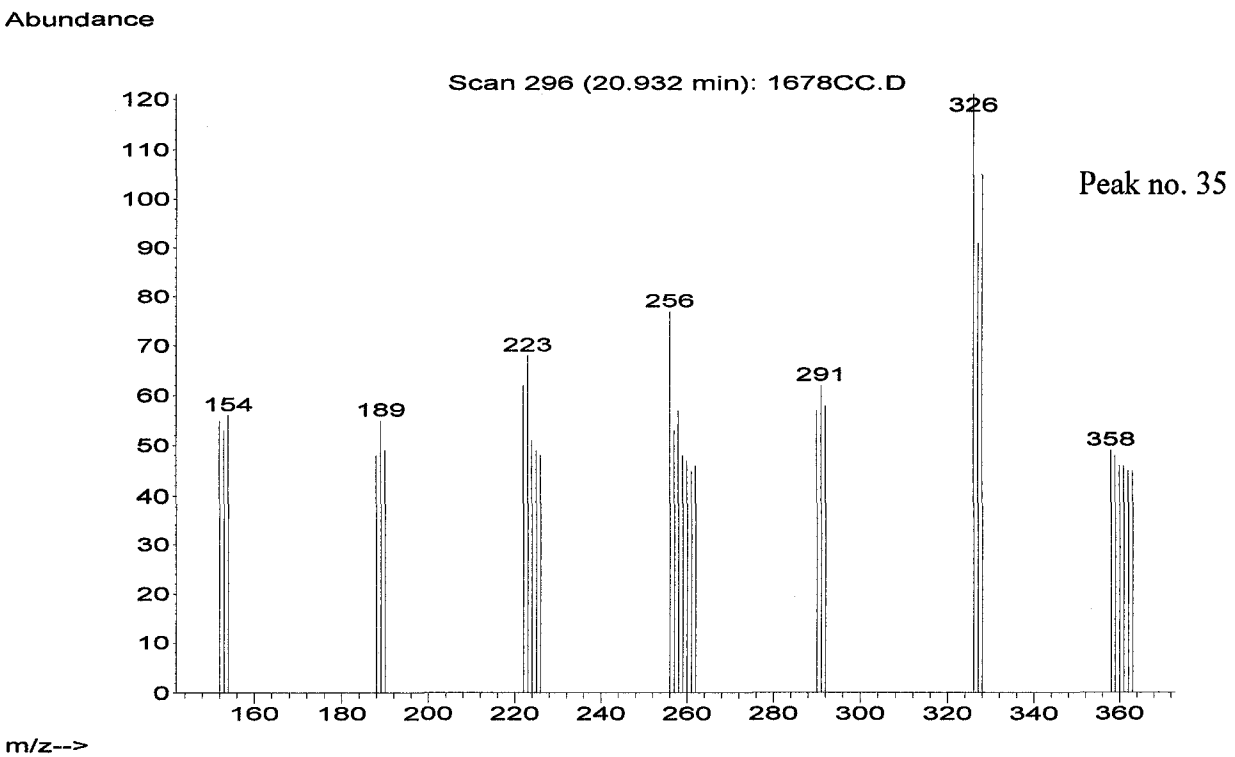

Abundance
Scan 271 (19.505 min): 1678CC.D
Peak no. 30
292
222
327
154
189
256
358
m/z-->

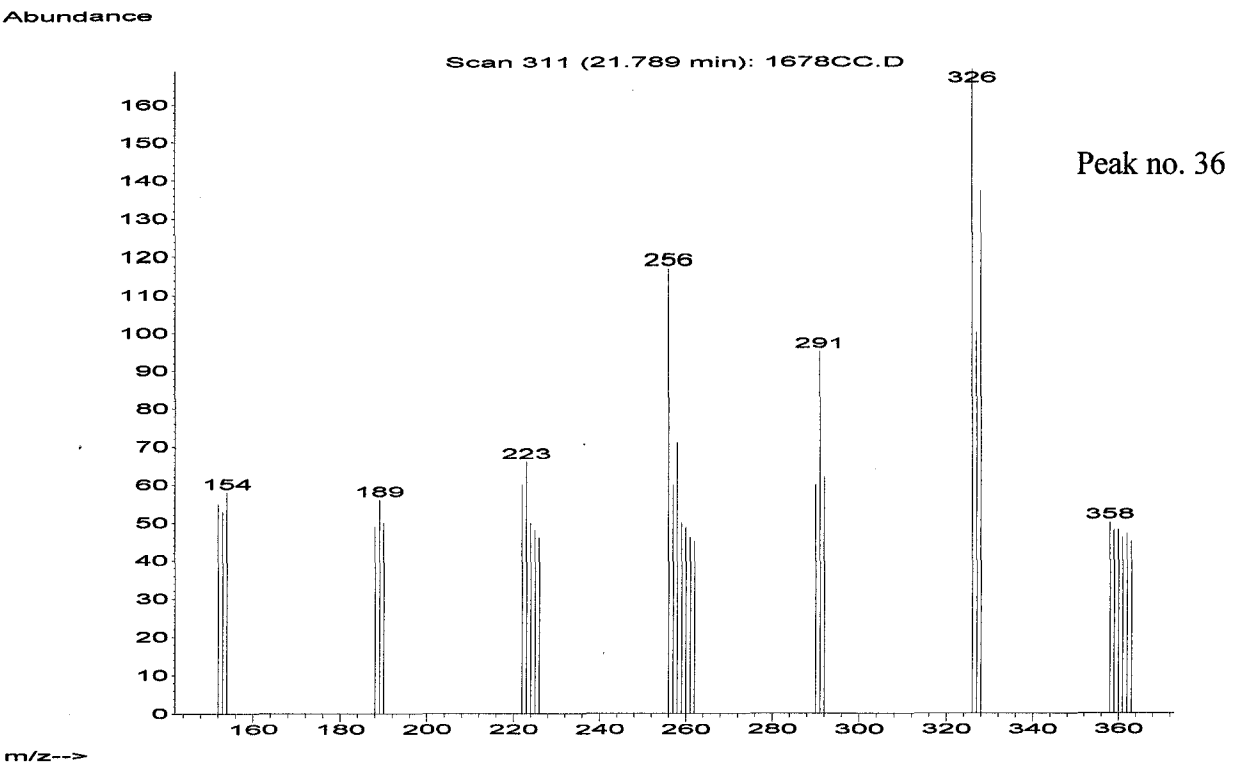

Abundance
Scan 277 (19.847 min): 1678CC.D
Peak no. 31
292
327
223
154
189
256
358
m/z-->


Abundance
Scan 281 (20.076 min): 1678CC.D
292
Peak no. 32
1000
900
800
700
600
500
400
300
200
100
0
222
256
154
189
327
358
160
180
200
220
240
260
280
300
320
340
360
m/z-->

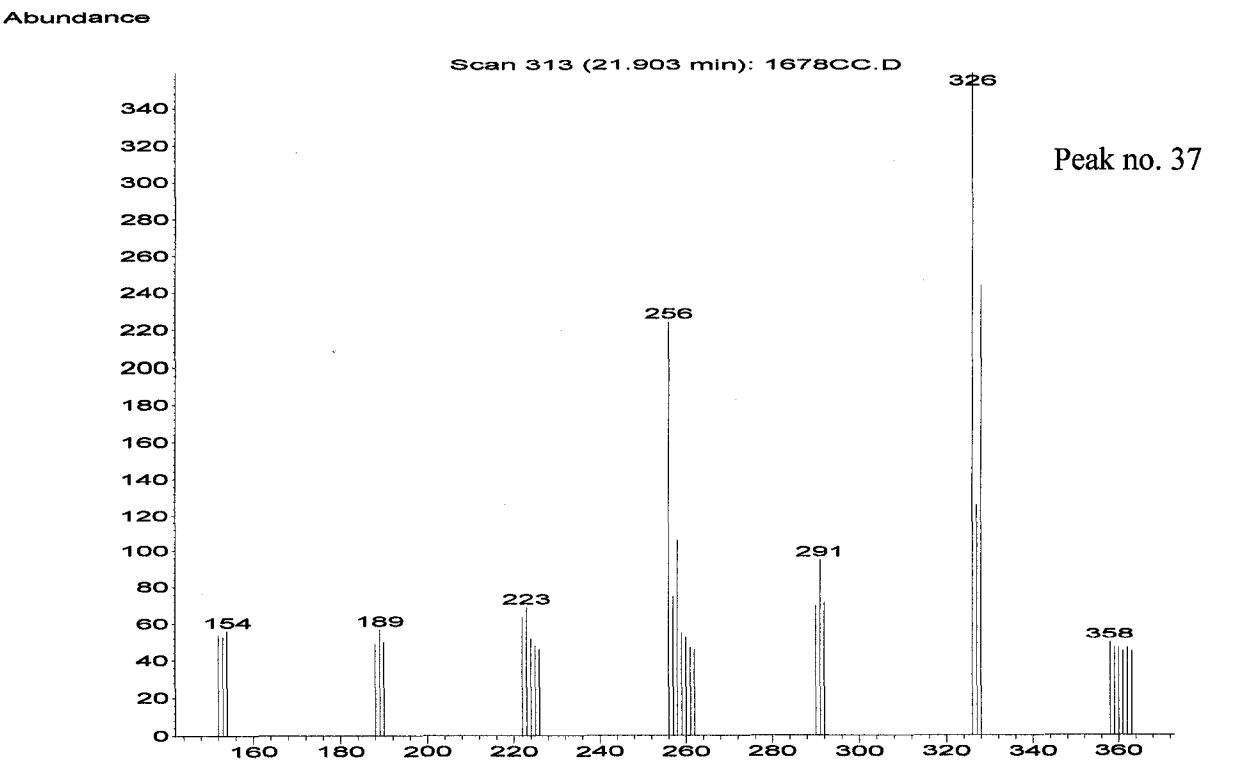

Abundance
Scan 285 (20.304 min): 1678CC.D
292
Peak no. 33
2000
1800
1600
1400
1200
1000
800
600
400
200
0
222
256
154
189
327
358
160
180
200
220
240
260
280
300
320
340
360
m/z-->


Abundance
Scan 288 (20.475 min): 1678CC.D
292
Peak no. 34
1600
1400
1200
1000
800
600
400
200
0
222
256
152
188
327
358
160 180 200 220 240 260 280 300 320 340 360
m/z-->

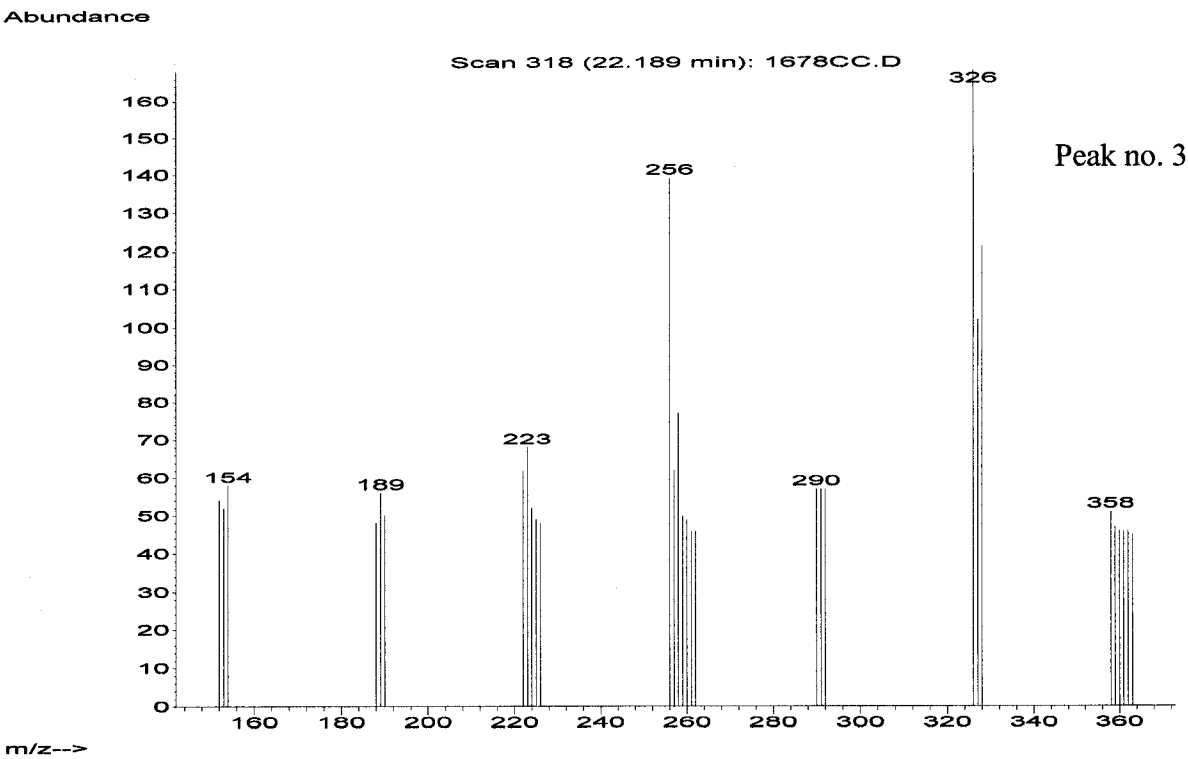

Abundance
Scan 296 (20.932 min): 1678CC.D
326
Peak no. 35
120
110
100
90
80
70
60
50
40
30
20
10
0
256
223
291
154
189
358
160 180 200 220 240 260 280 300 320 340 360
m/z-->

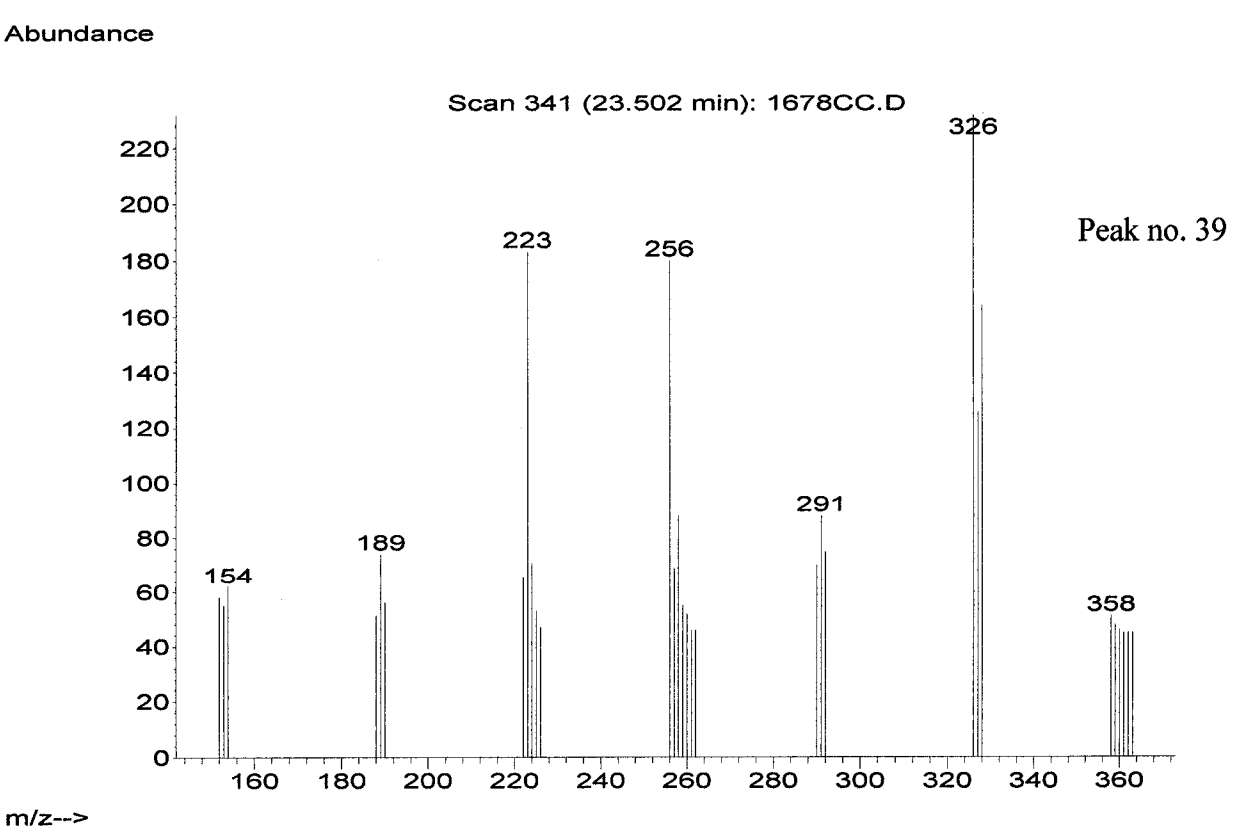


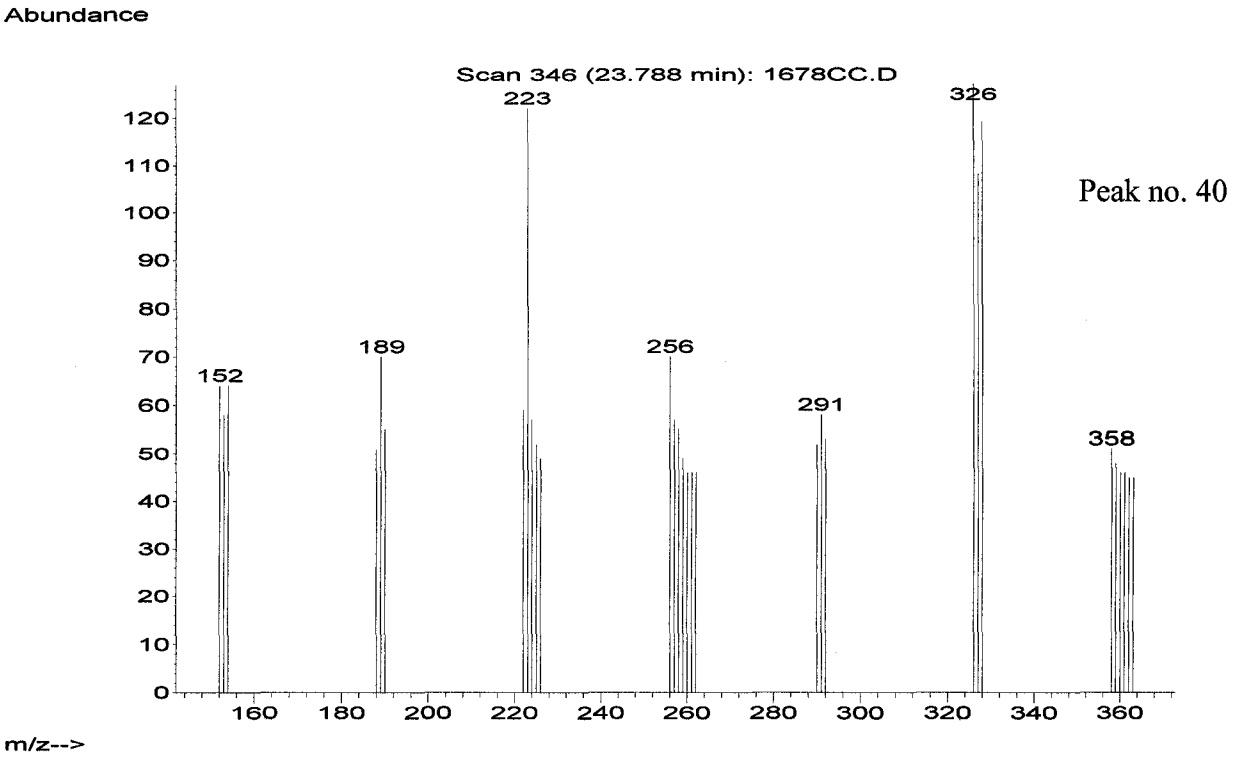
Peak no. 36

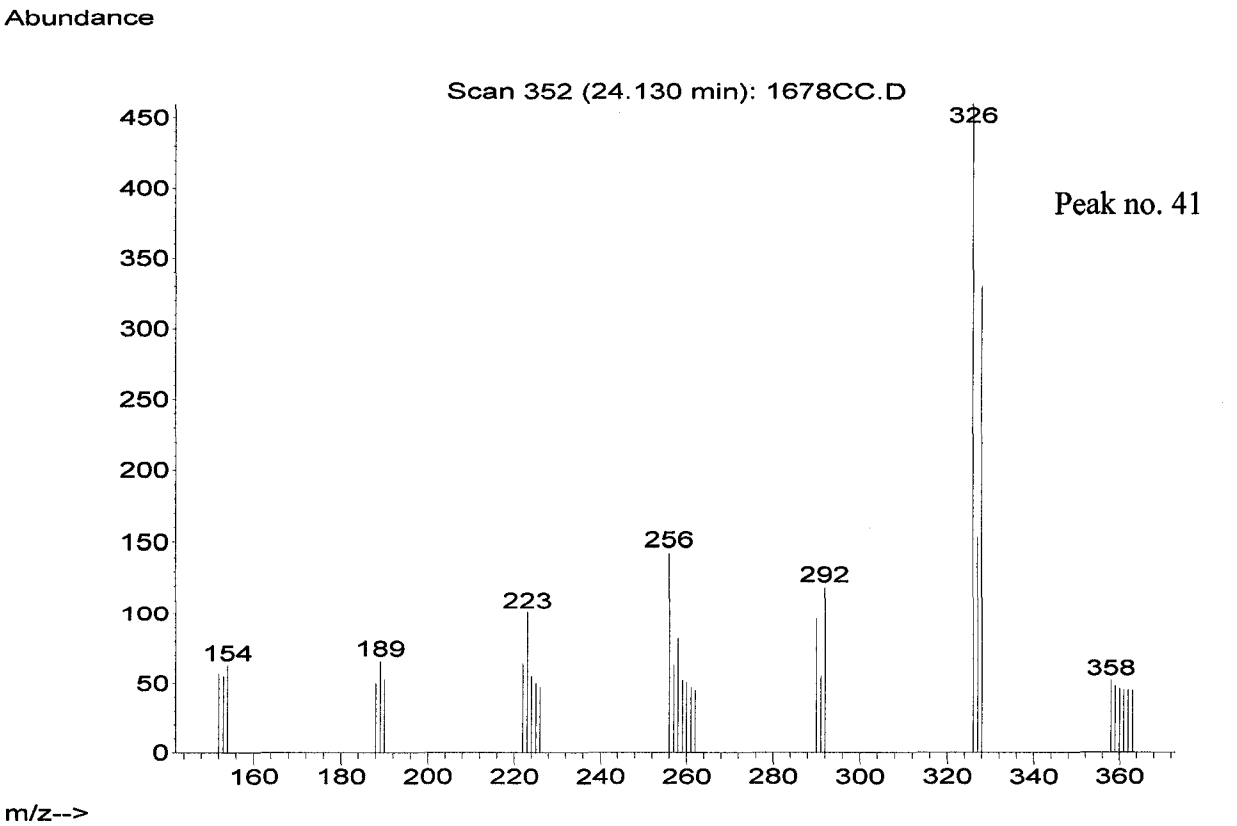


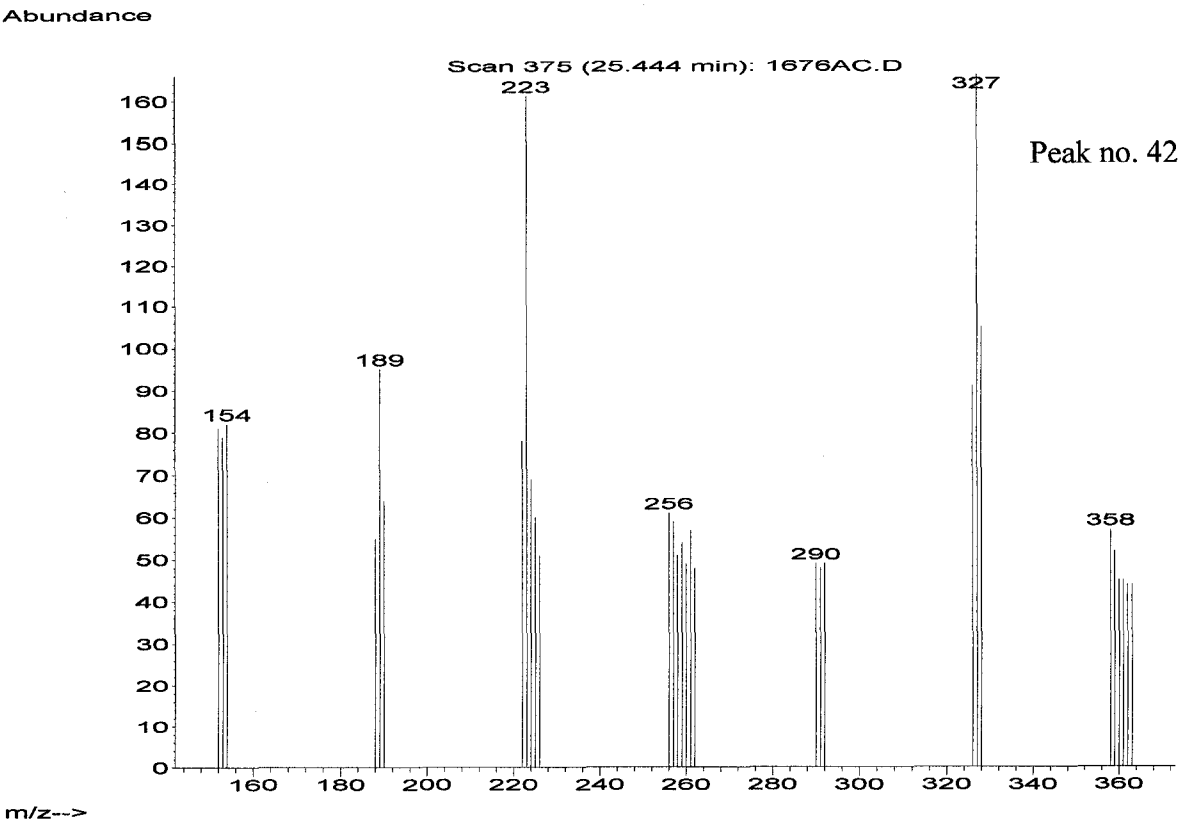
Peak no. 37


Abundance
Scan 318 (22.189 min): 1678CC.D
326
256
223
154
189
290
358
Peak no. 38
m/z-->

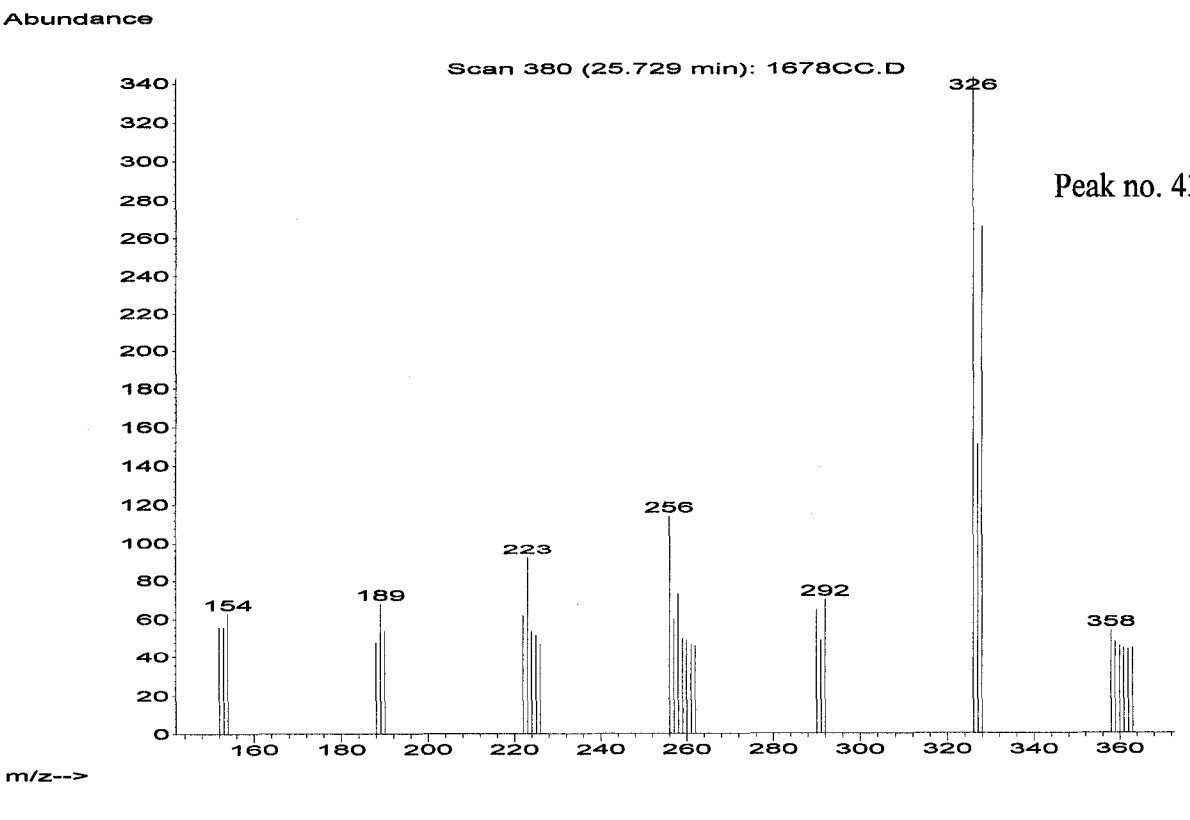

Abundance
Scan 341 (23.502 min): 1678CC.D
326
223
256
291
189
154
358
Peak no. 39
m/z-->


Abundance
Scan 346 (23.788 min): 1678CC.D
223
326
189
256
152
291
358
Peak no. 40
m/z-->

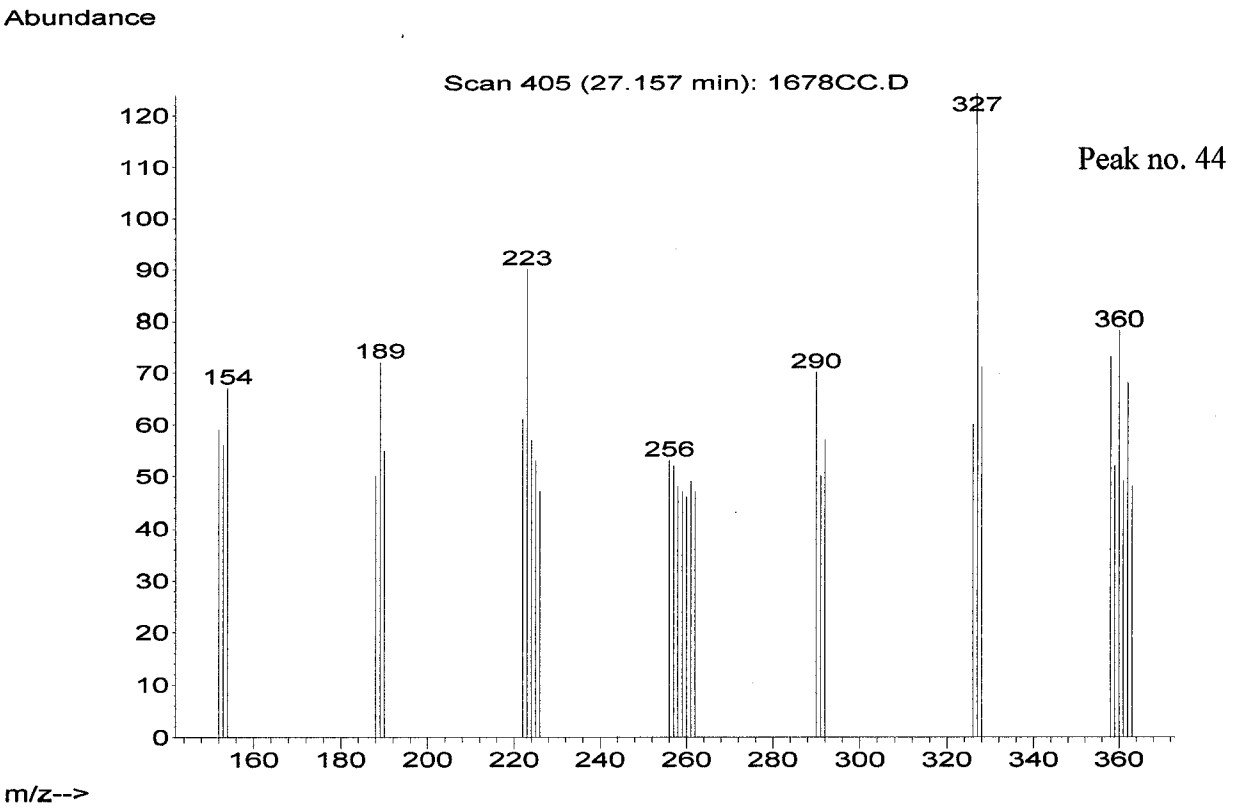

Abundance
Scan 352 (24.130 min): 1678CC.D
326
256
292
223
154
189
358
Peak no. 41
m/z-->

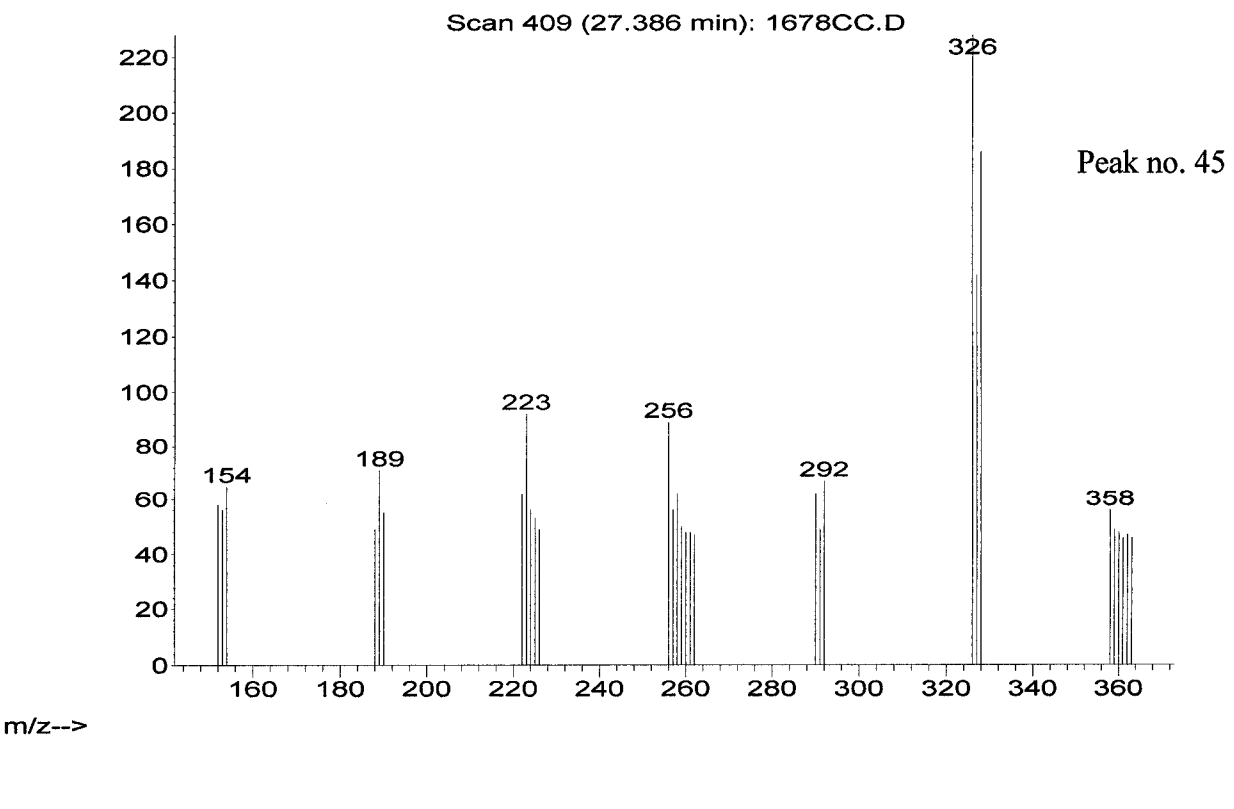


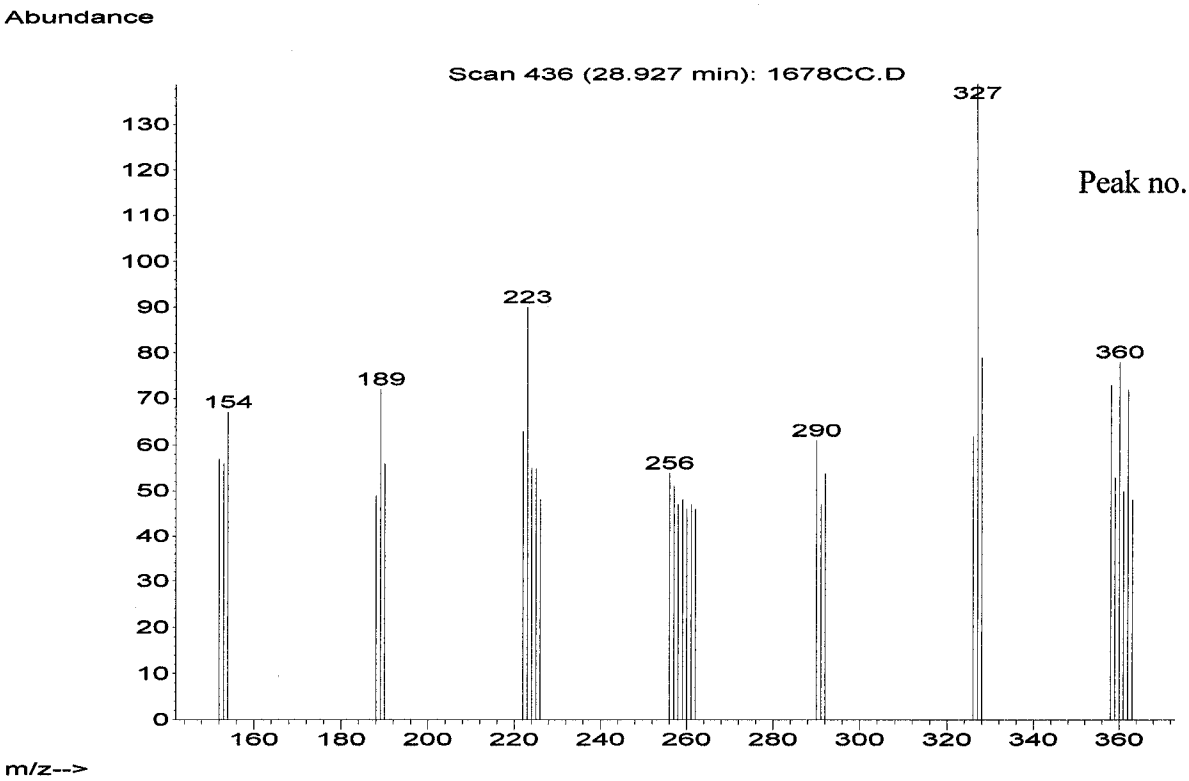
Peak no. 42



Peak no. 43


Abundance
Scan 405 (27.157 min): 1678CC.D
Peak no. 44
154
189
223
256
290
327
360
m/z-->

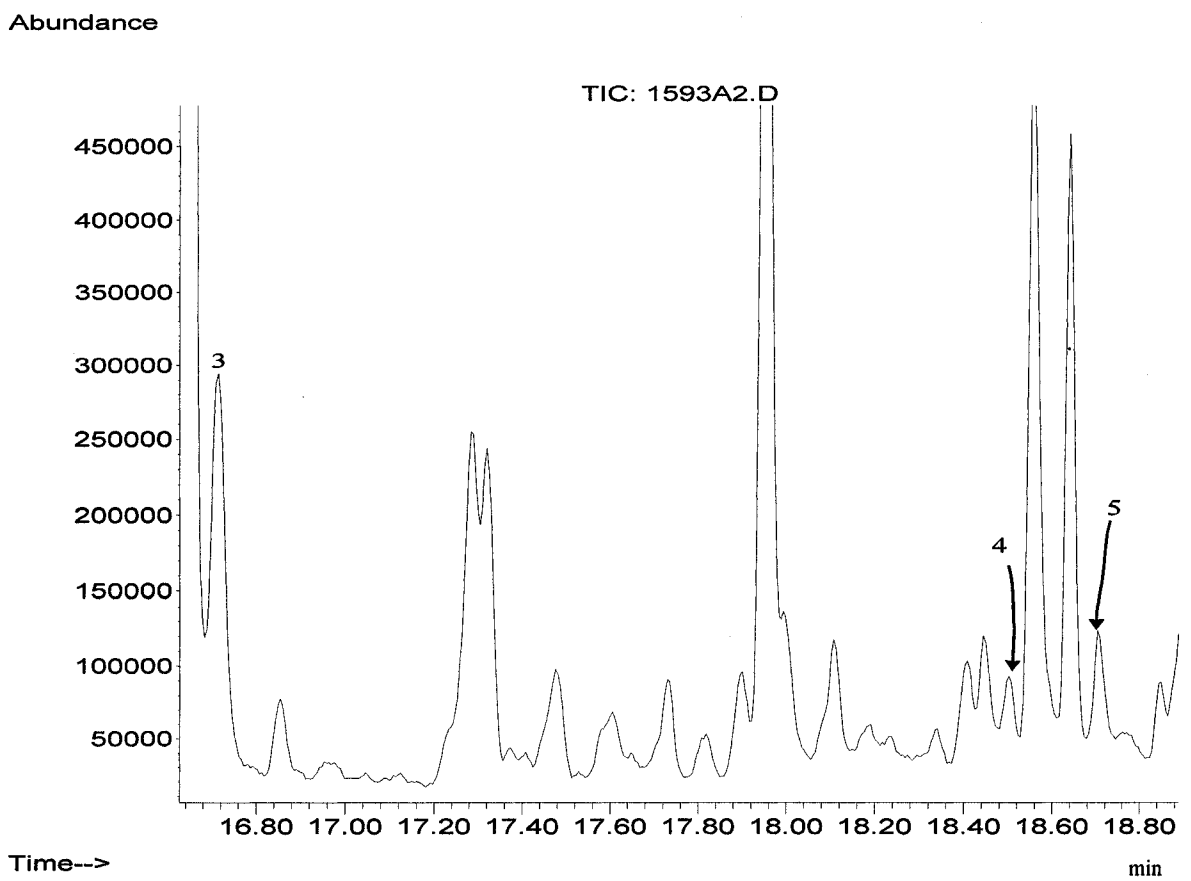

Abundance
Scan 409 (27.386 min): 1678CC.D
Peak no. 45
154
189
223
256
292
326
358
m/z-->


Abundance
Scan 436 (28.927 min): 1678CC.D
327
130
120
110
100
90
80
70
60
50
40
30
20
10
0
223
360
189
154
290
256
160
180
200
220
240
260
280
300
320
340
360
m/z-->

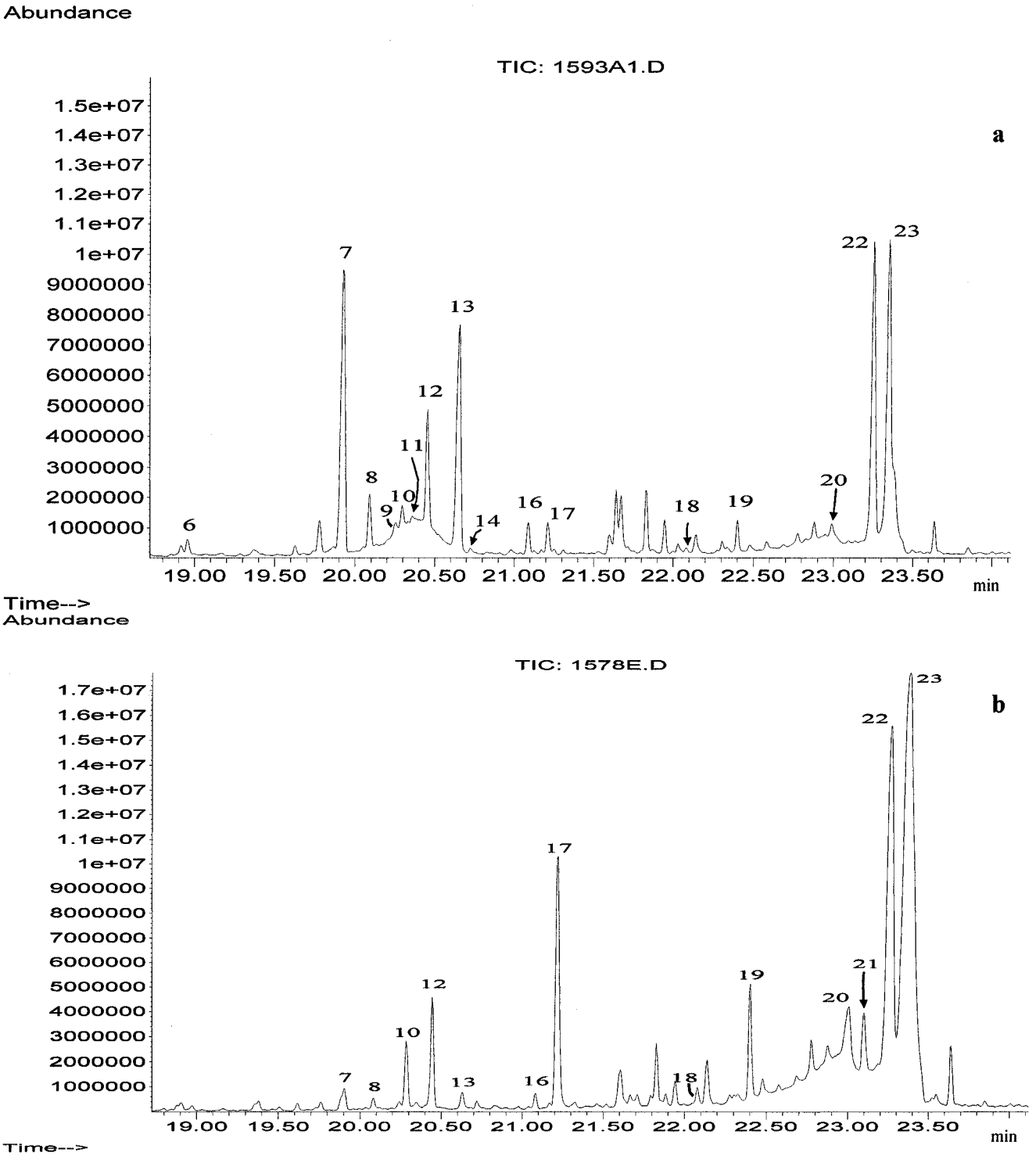
Peak no. 46

Appendix B

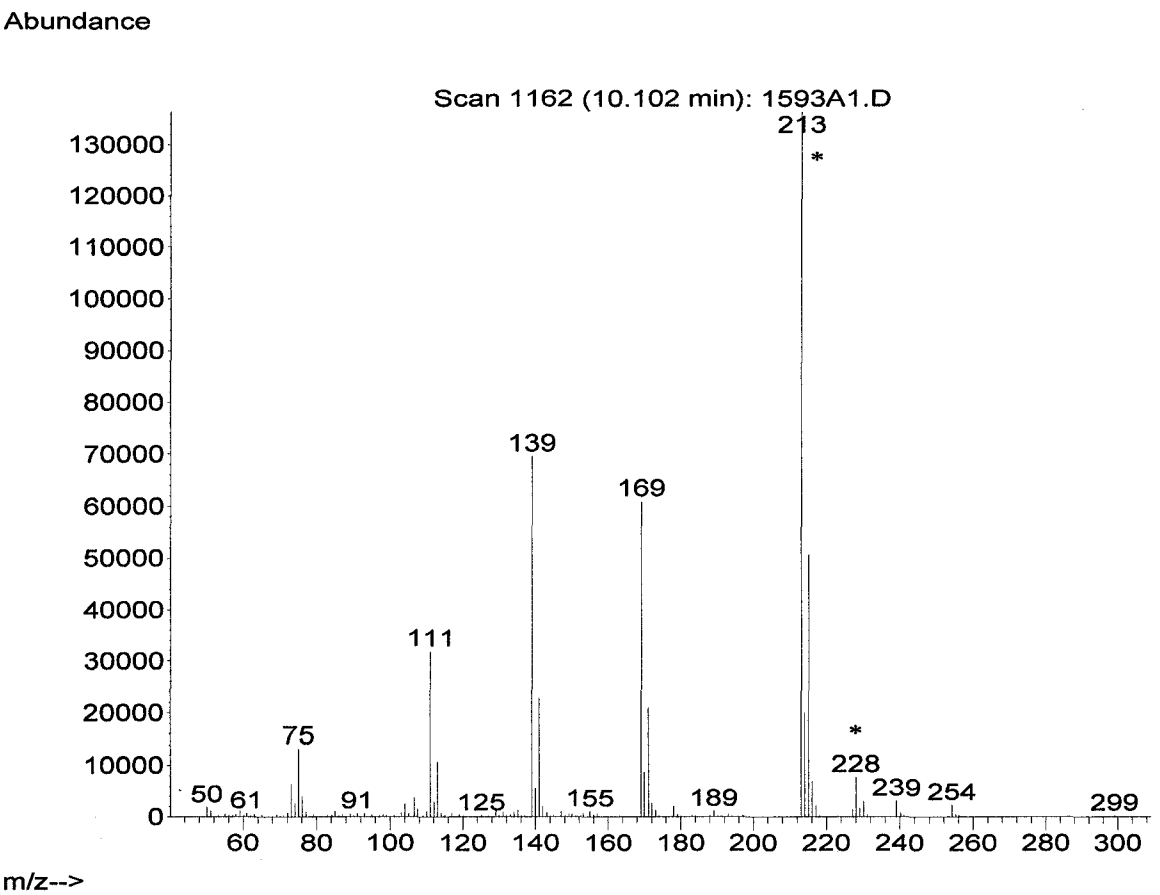
Gas chromatogram and mass spectra of TMS-derivatized metabolic products detected in acidic and neutral extracts of cultures of biphenyl-grown cells of *Hydrogenophaga* sp. IA3-A incubated with 2,4'-dichlorobiphenyl.



A section of the TIC of acidic and neutral extracts of cultures incubated with 2,4'-diCB at 10 °C for 12 h showing compounds in **peak nos. 3, 4, and 5**.

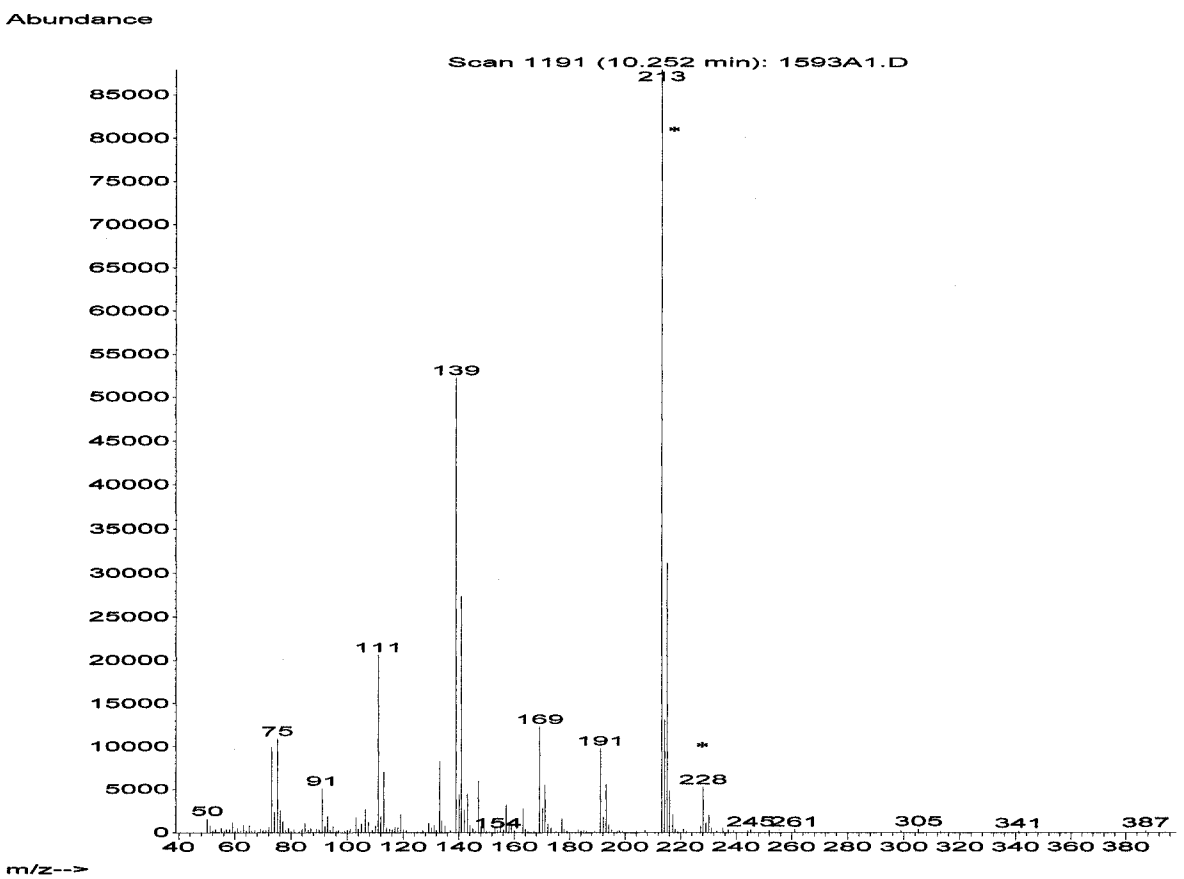


A section of the TIC of acidic and neutral extracts of cultures incubated with 2,4'-diCB at 10 °C for 4 h (a) and 48 h (b) showing compounds in **peak nos. 6 - 23**. Peak no. 15 is not shown due to very low level of detection, but the mass spectrum is shown below.

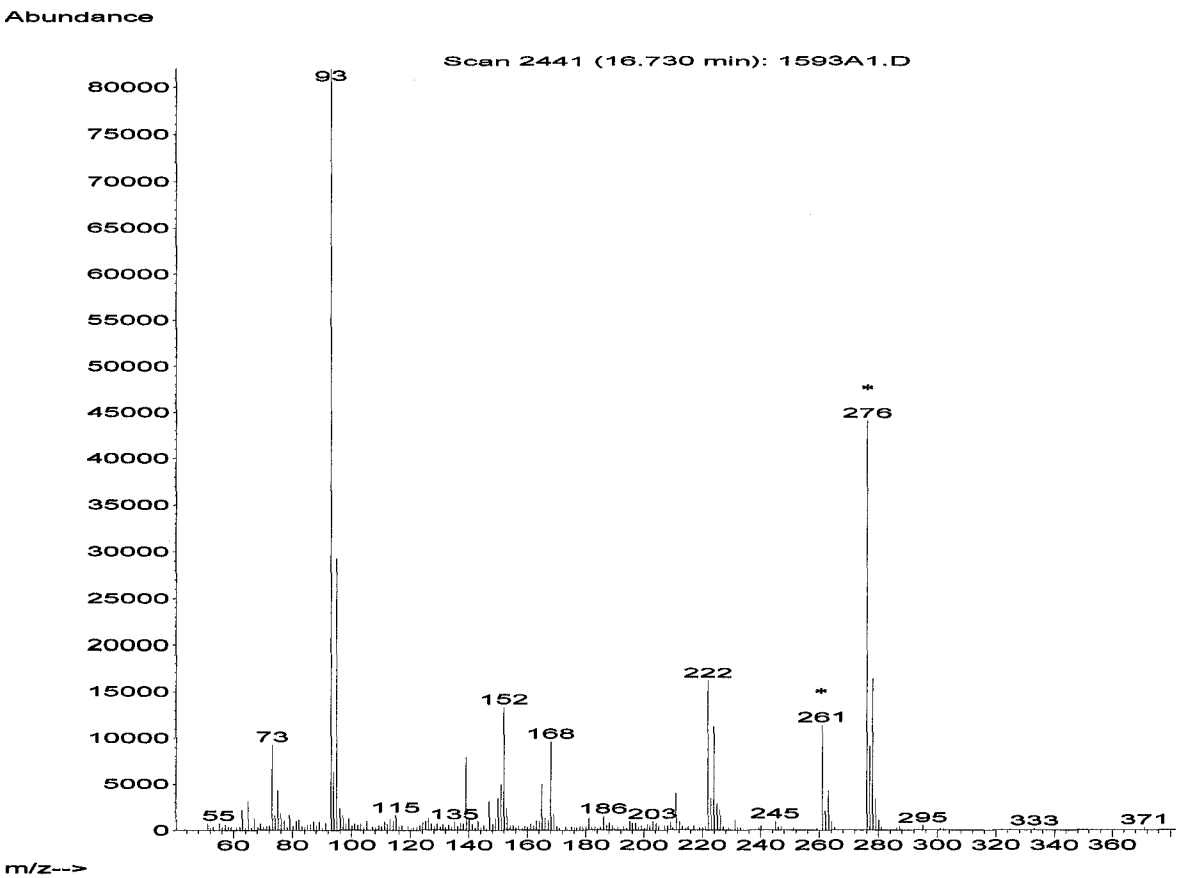


Mass spectrum of the compound in **peak no. 1** identified as 4-chlorobenzoate.

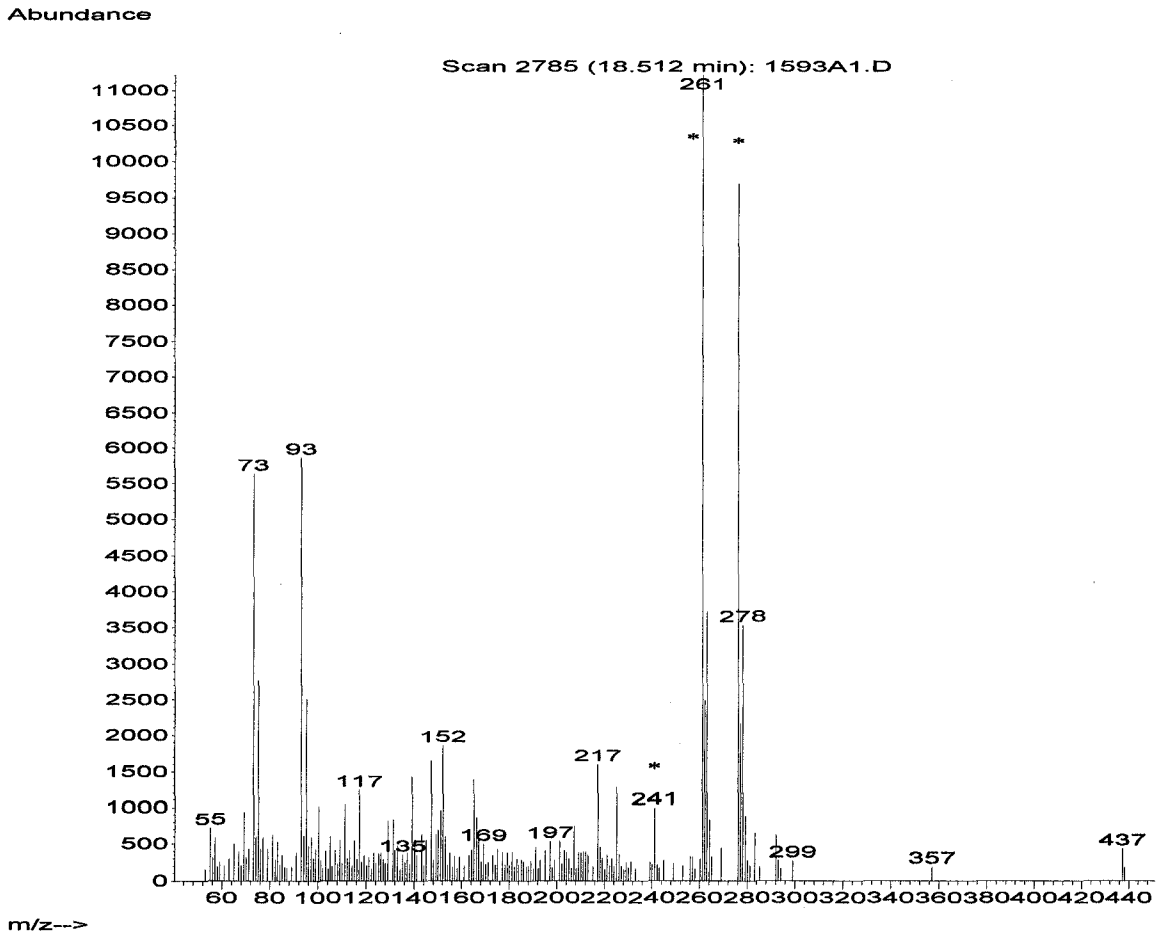
Characteristic ions are indicated with an asterisk.



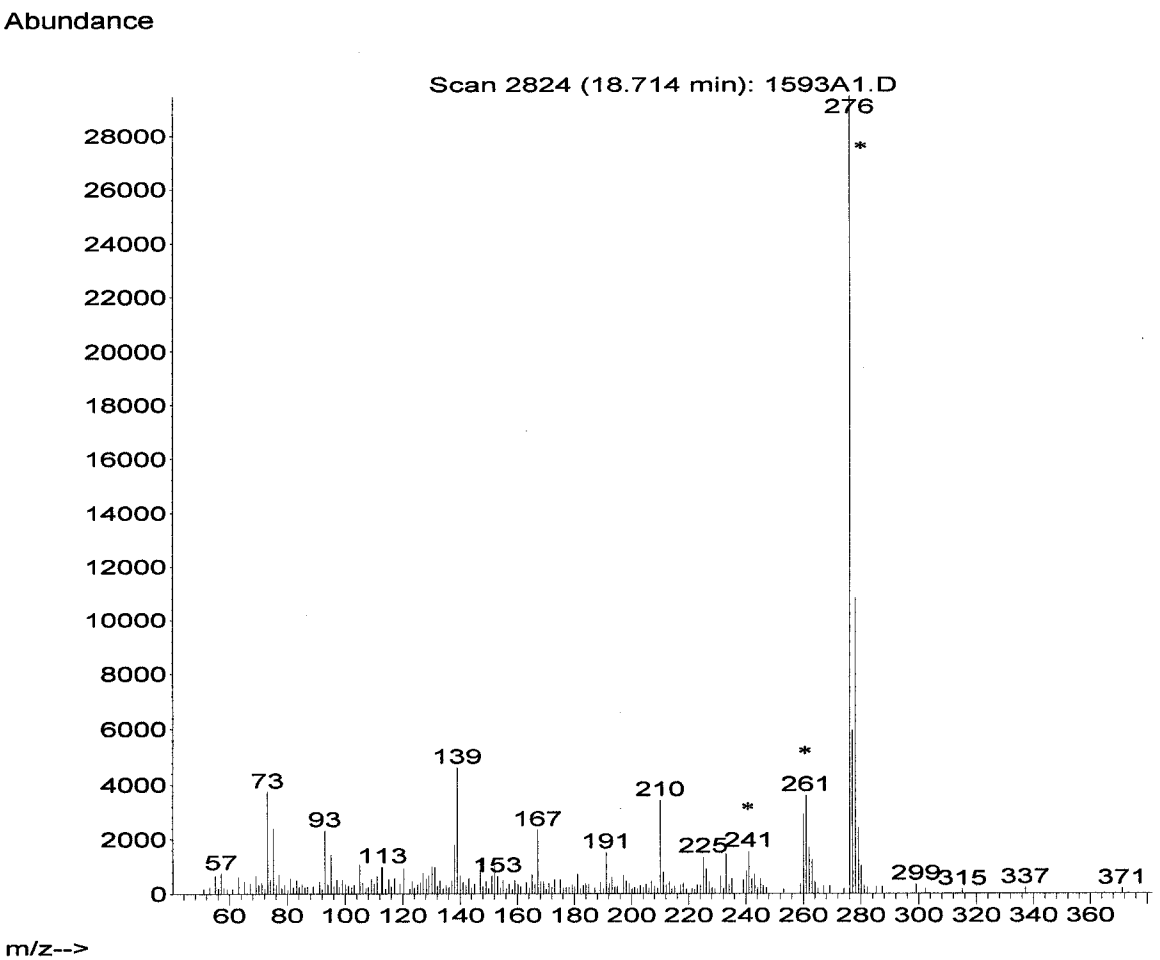
Mass spectrum of the compound in **peak no. 2** identified as 2-chlorobenzoate



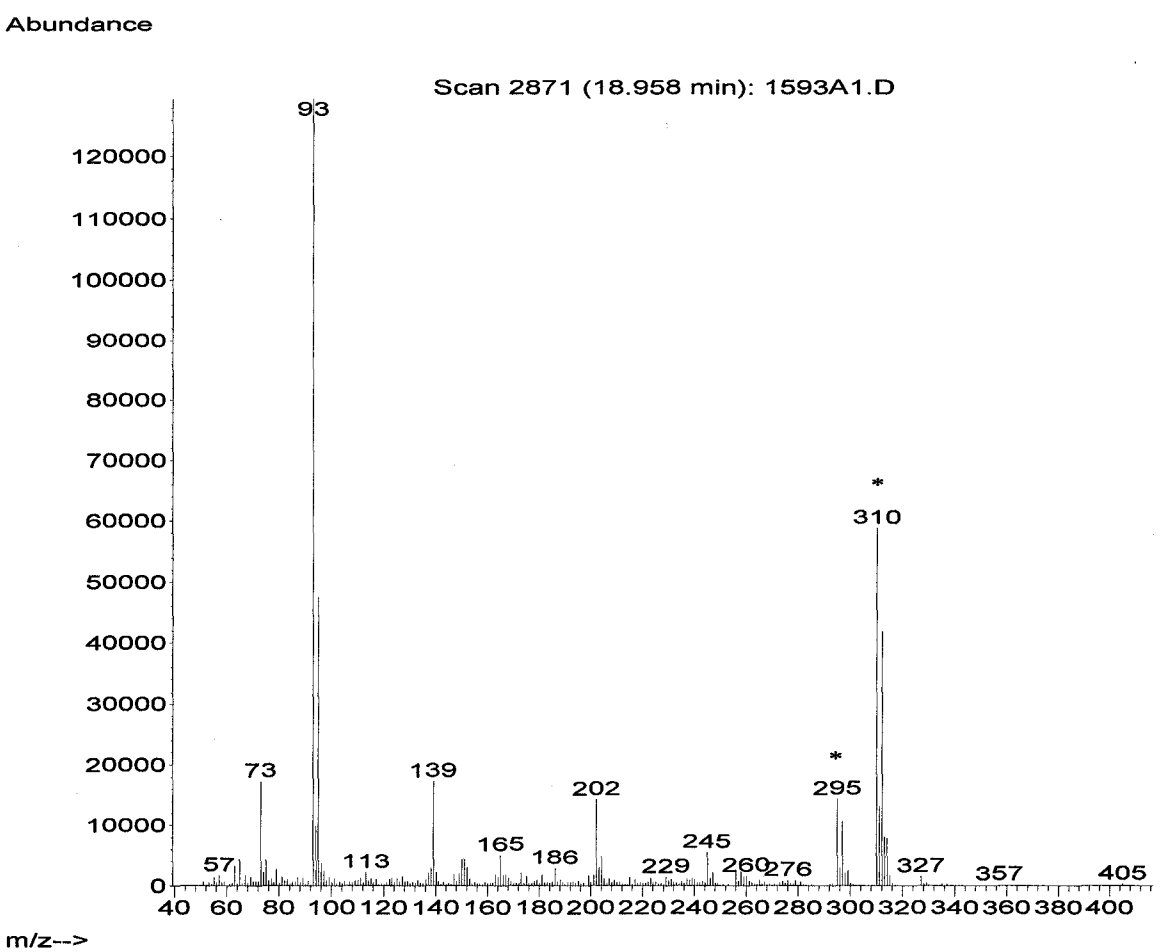
Mass spectrum of the compound in **peak no. 3** proposed to be monochlorohydroxybiphenyl.



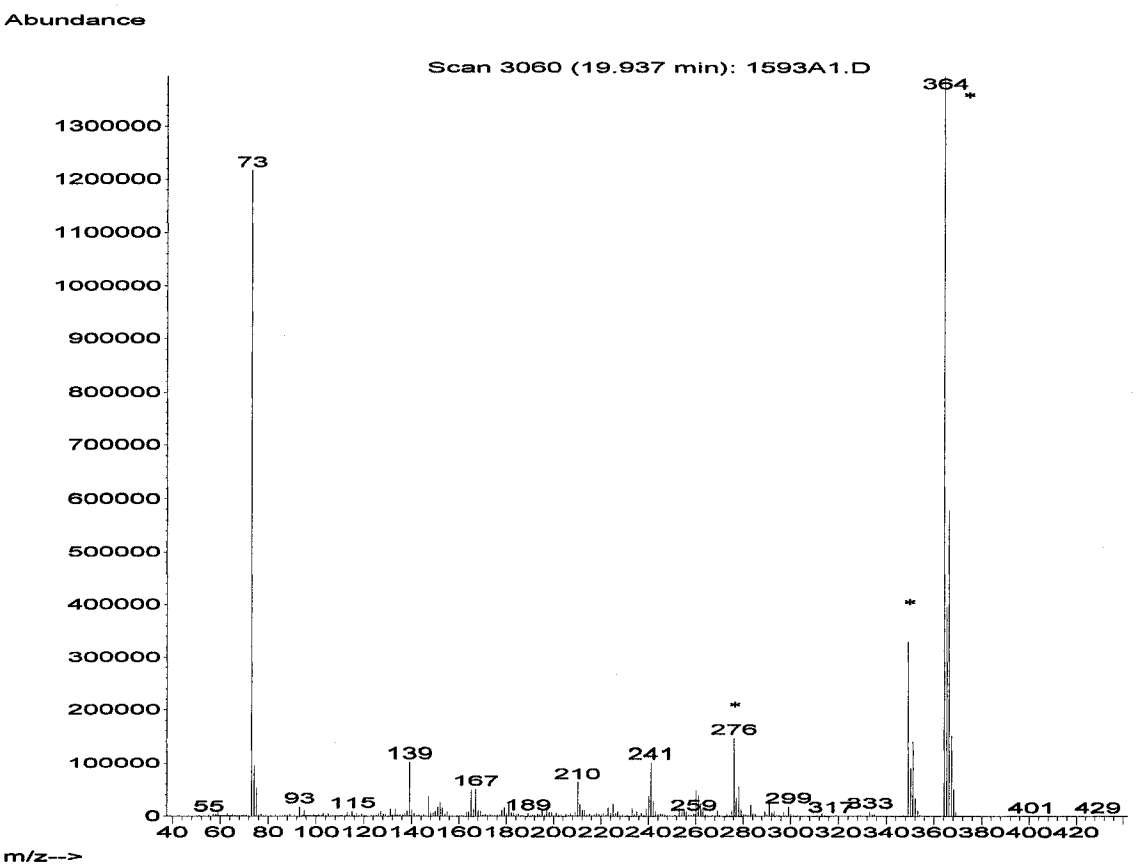
Mass spectrum of the compound in **peak no. 4** proposed to be monochlorohydroxybiphenyl



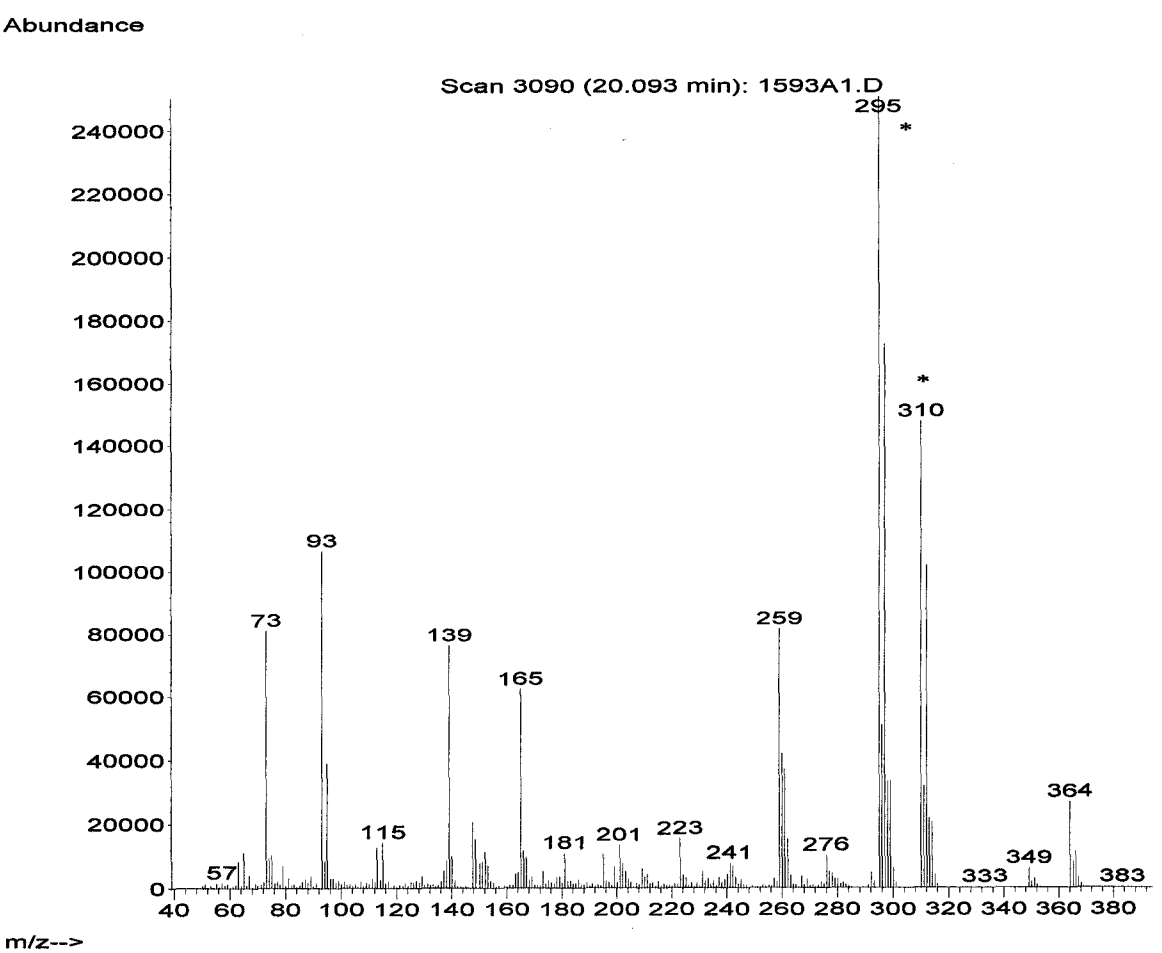
Mass spectrum of the compound in **peak no. 5** proposed to be monochlorohydroxybiphenyl



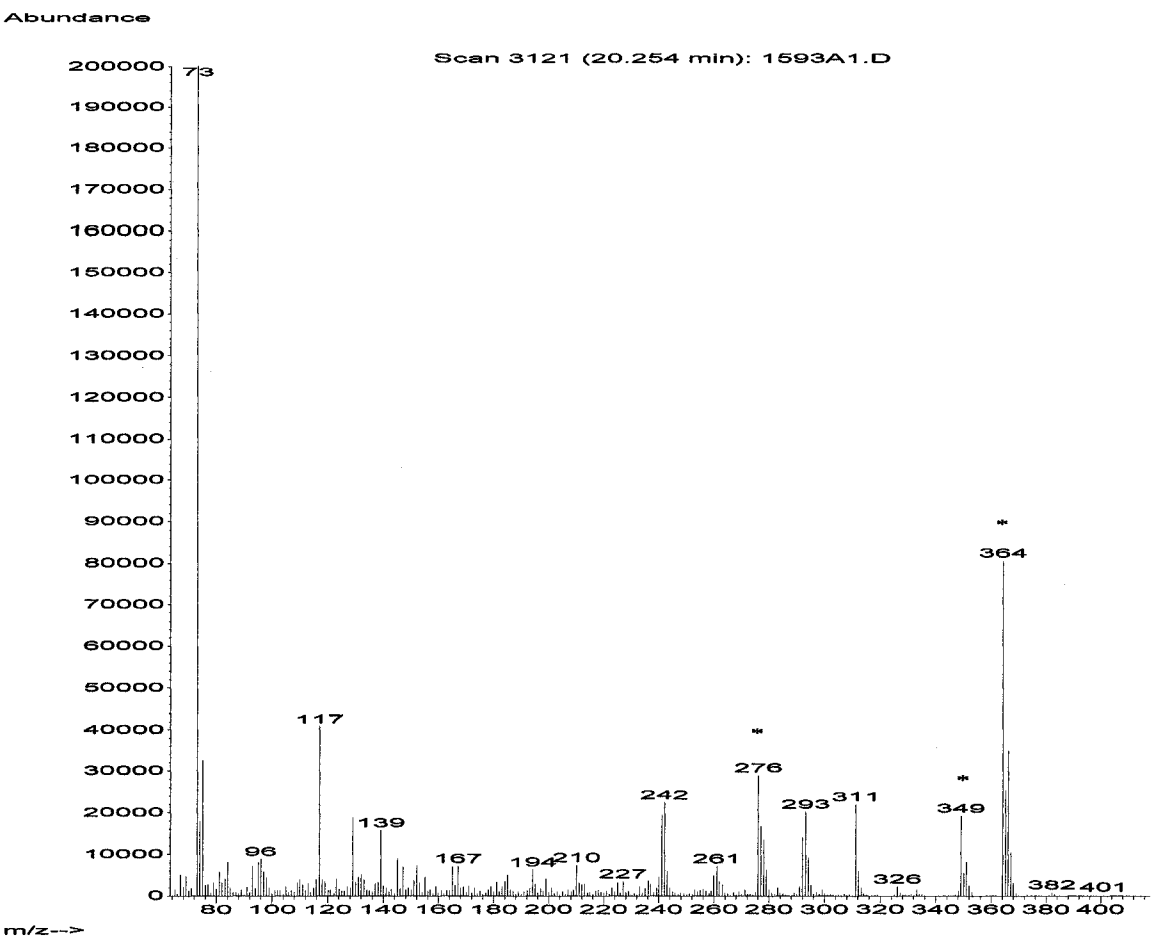
Mass spectrum of the compound in **peak no. 6** proposed to be 2,4'-dichlorohydroxybiphenyl



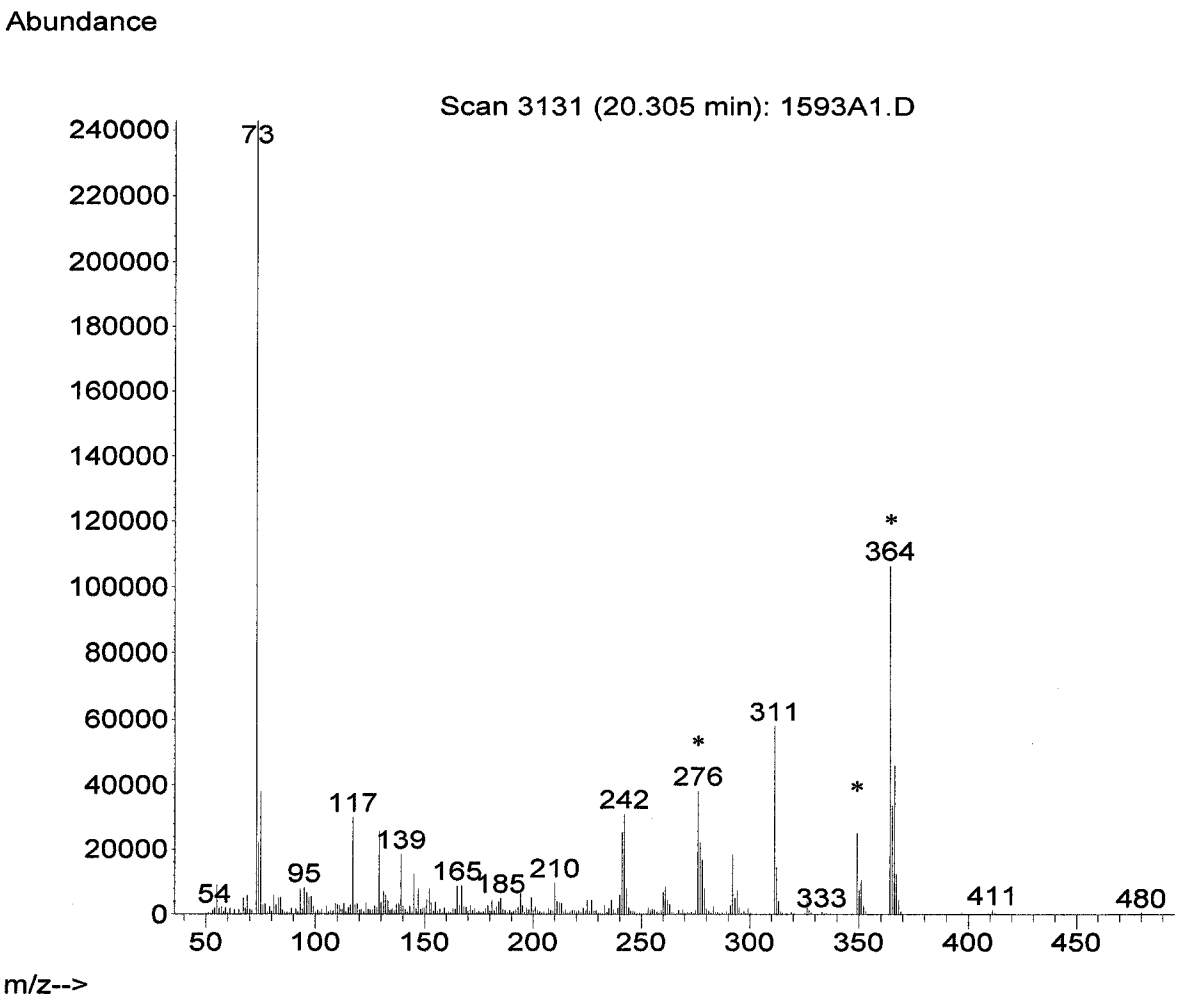
Mass spectrum of the compound in **peak no. 7** proposed to be monochlorodihydroxybiphenyl



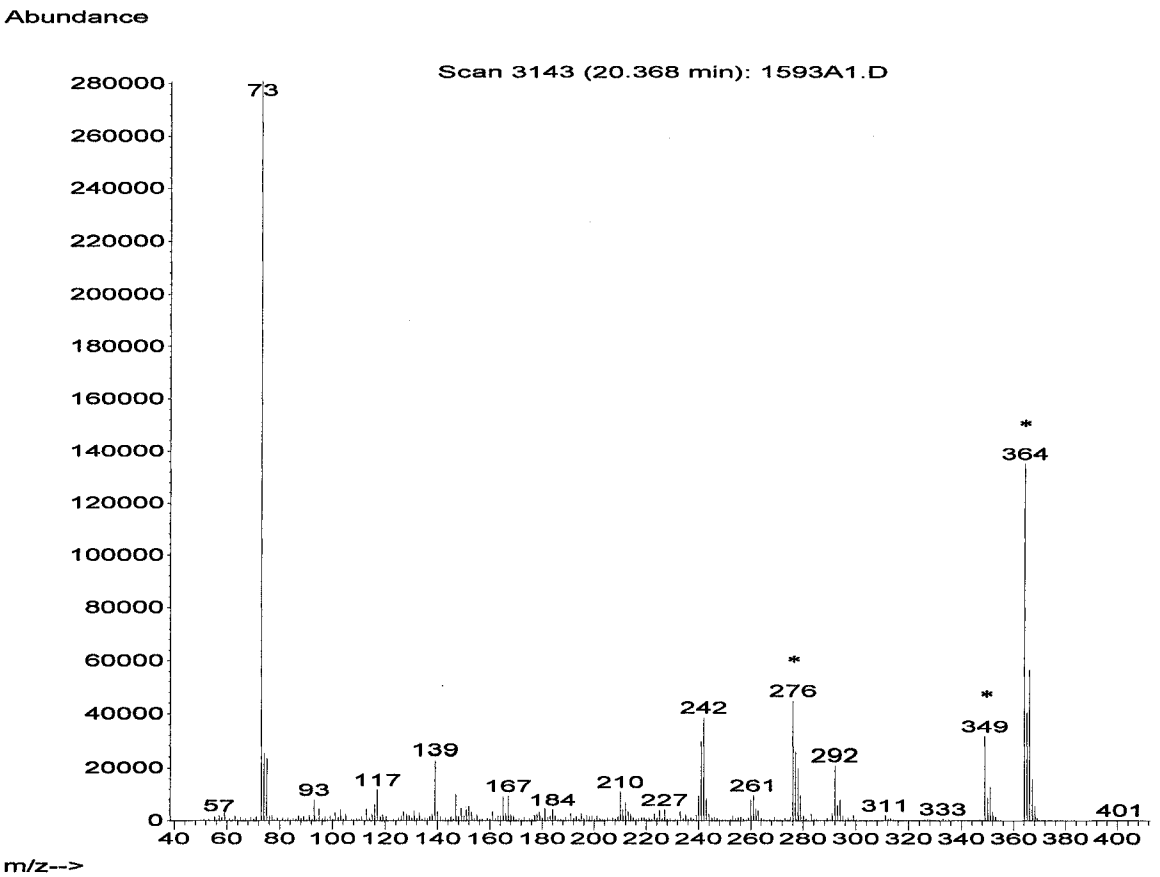
Mass spectrum of the compound in **peak no. 8** proposed to be 2,4'-dichlorohydroxybiphenyl



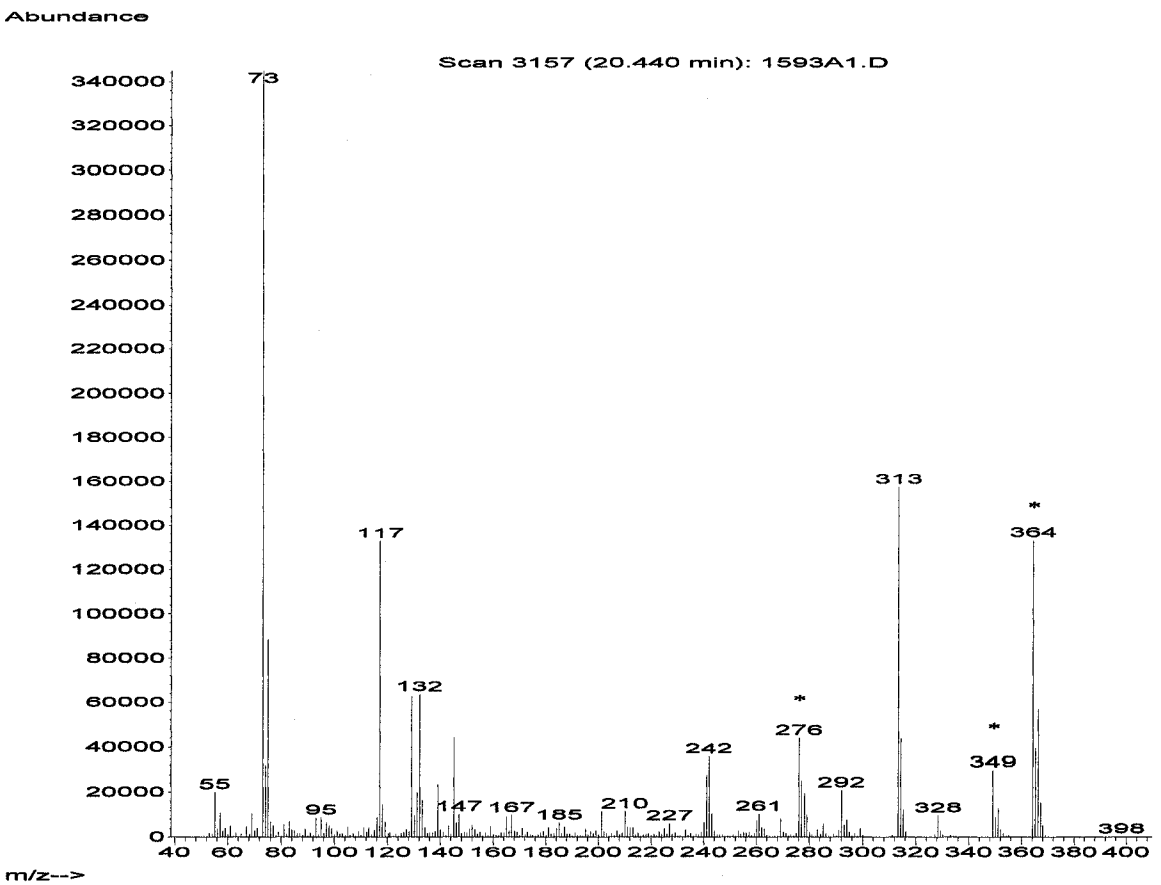
Mass spectrum of the compound in **peak no. 9** proposed to be monochlorodihydroxybiphenyl



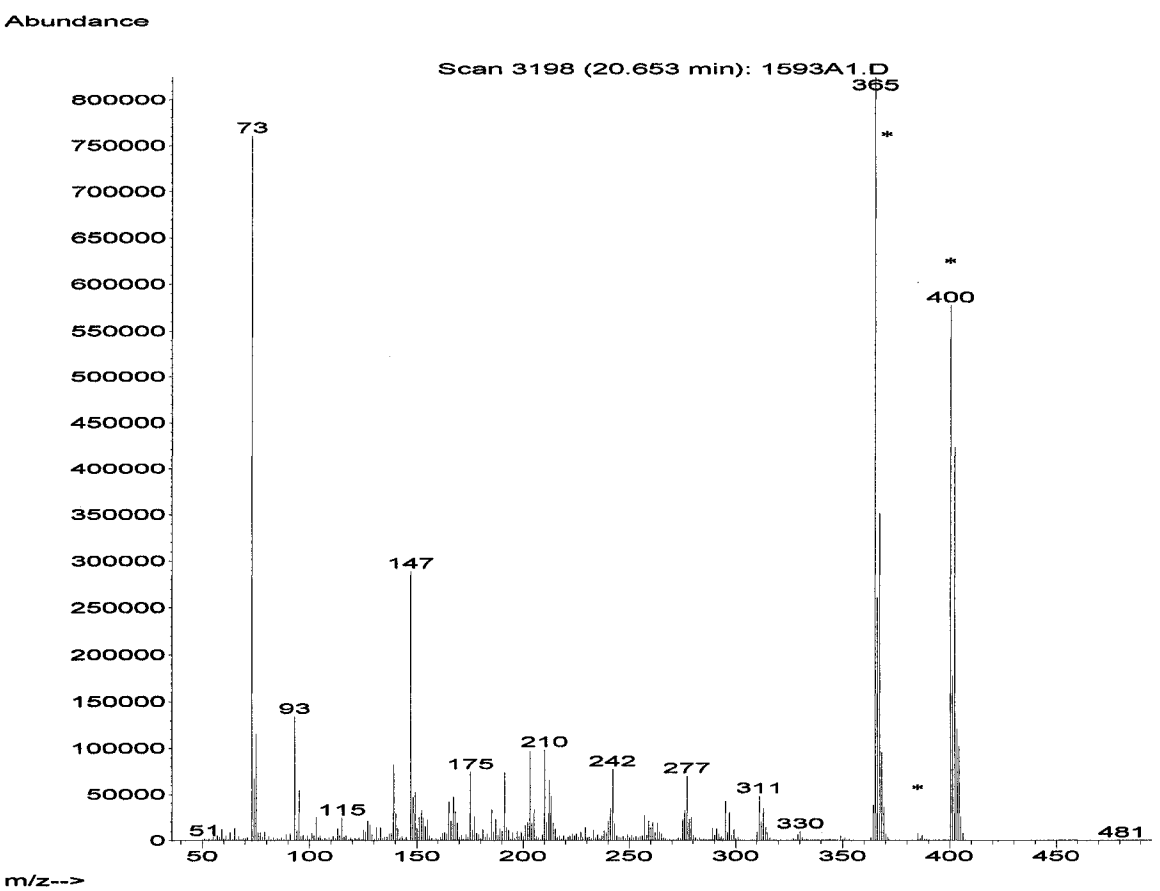
Mass spectrum of the compound in **peak no. 10** proposed to be monochlorodihydroxybiphenyl



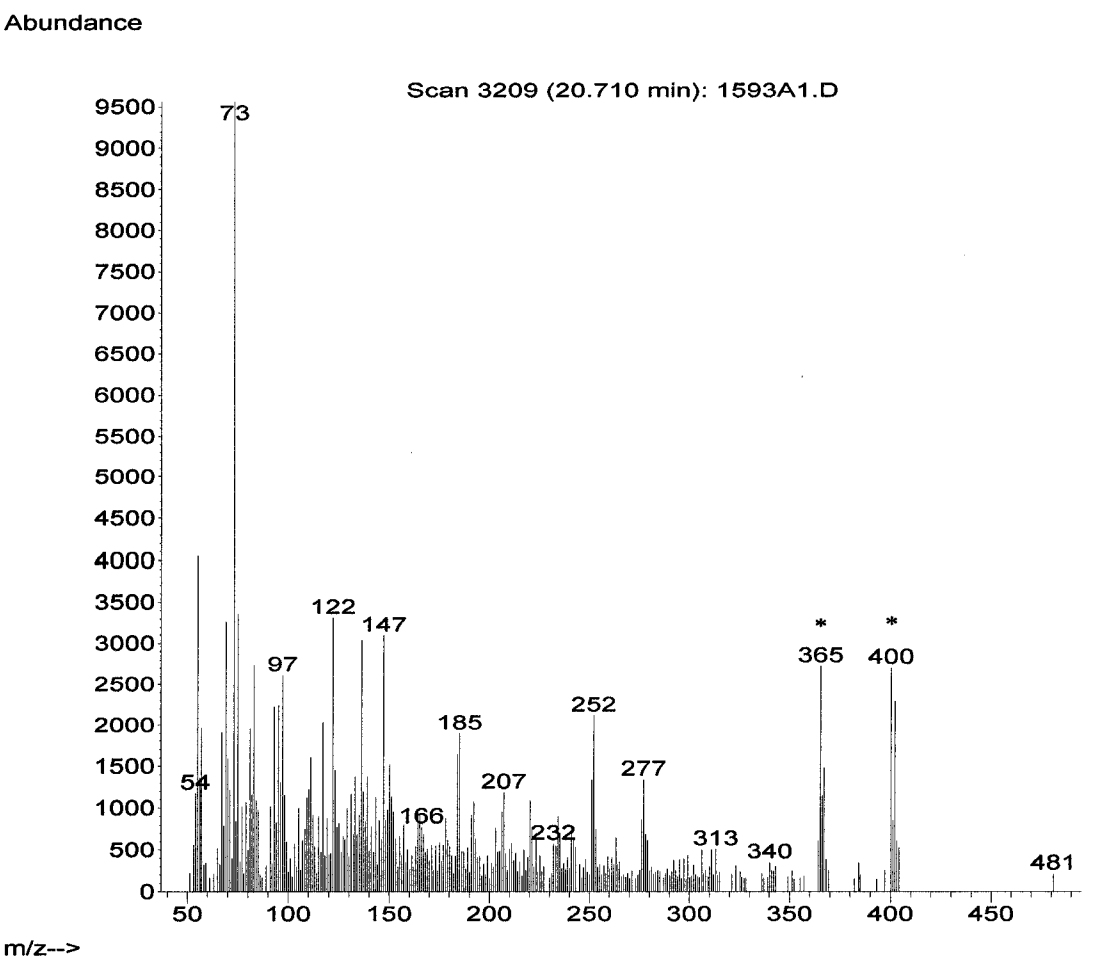
Mass spectrum of the compound in **peak no. 11** proposed to be monochlorodihydroxybiphenyl



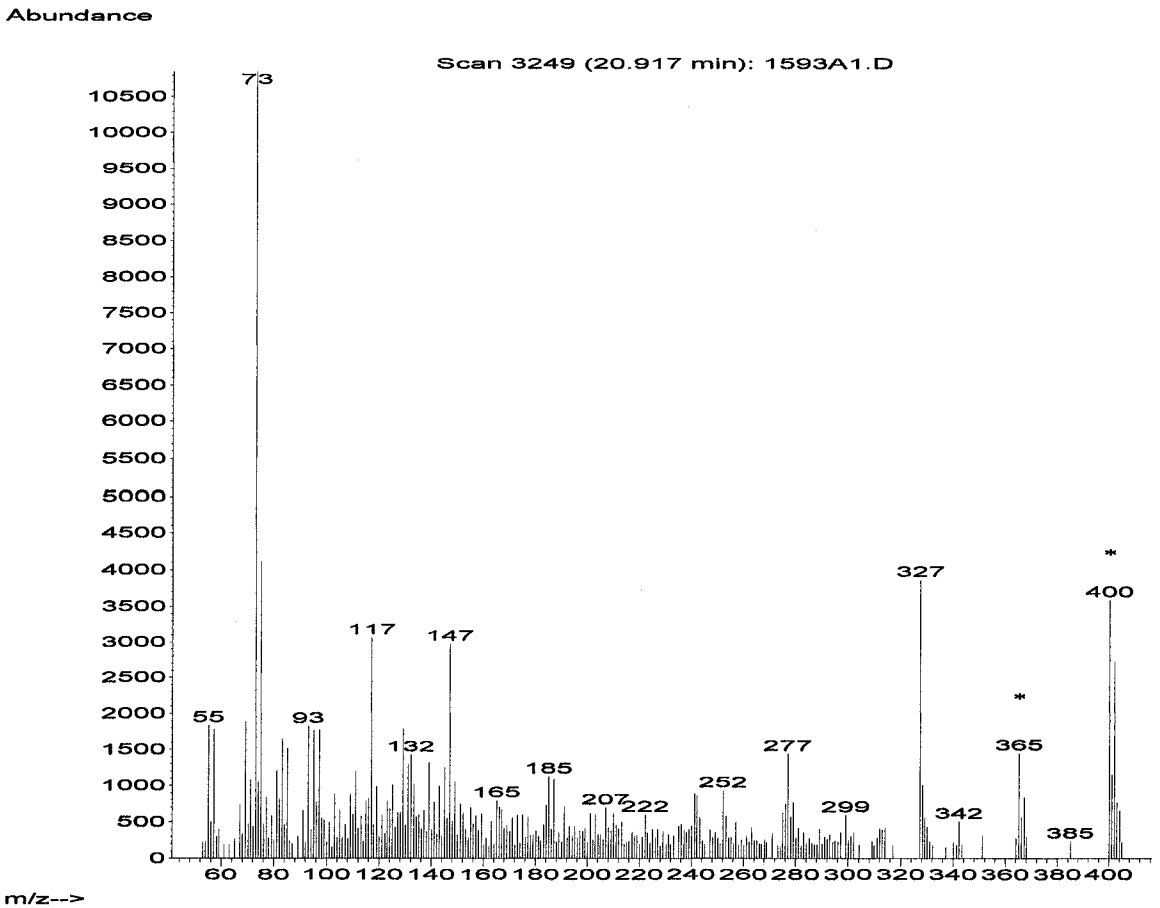
Mass spectrum of the compound in **peak no. 12** proposed to be monochlorodihydroxybiphenyl



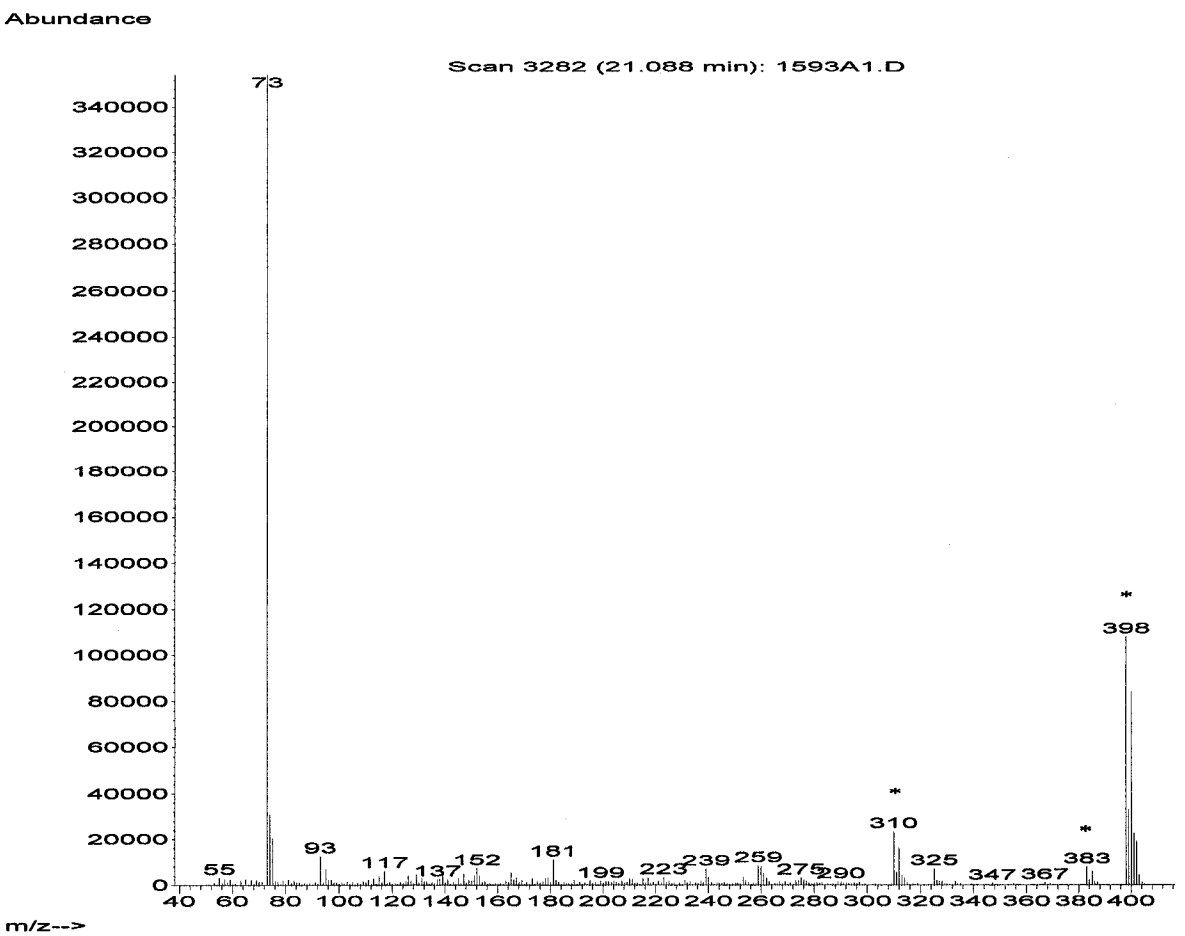
Mass spectrum of the compound in **peak no. 13** proposed to be dichloro-dihydrodiol



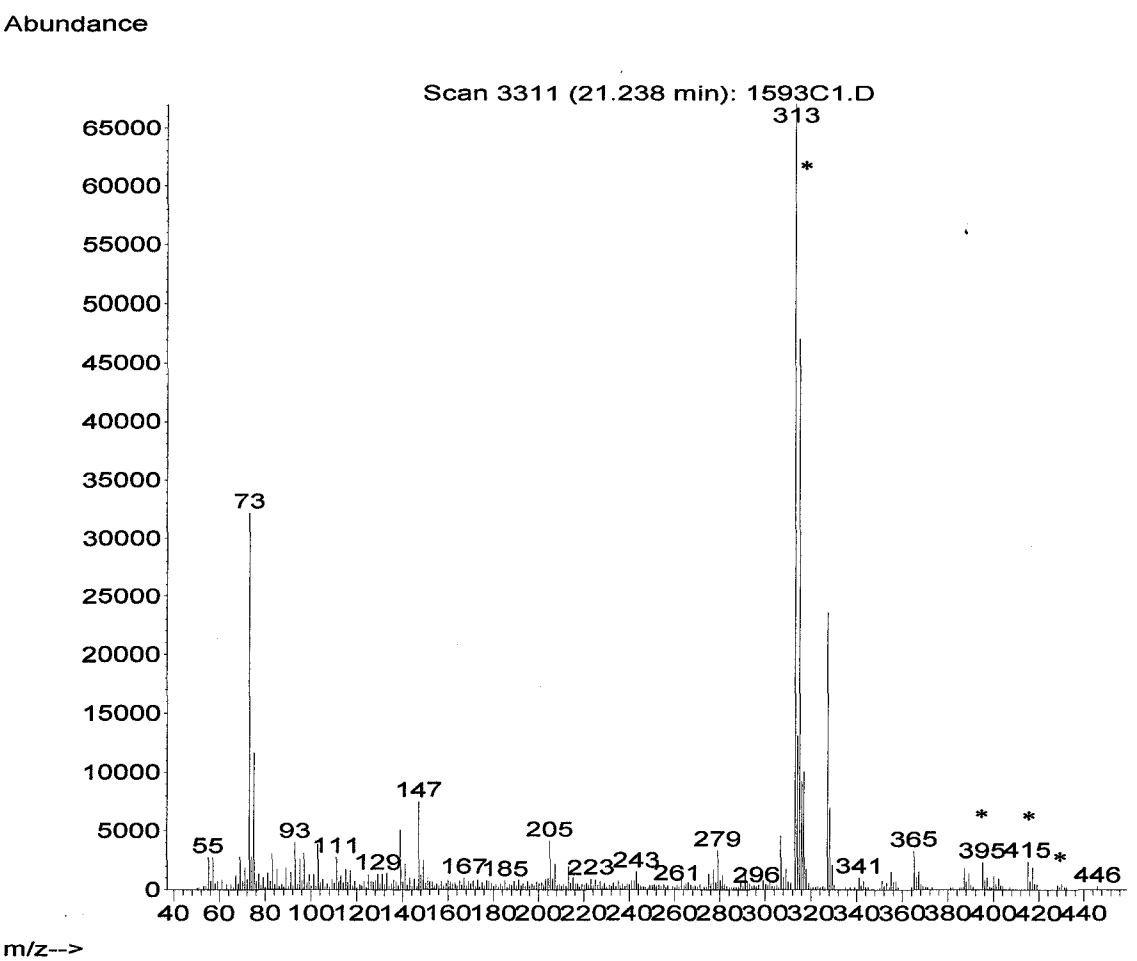
Mass spectrum of the compound in **peak no. 14** proposed to be dichloro-dihydrodiol



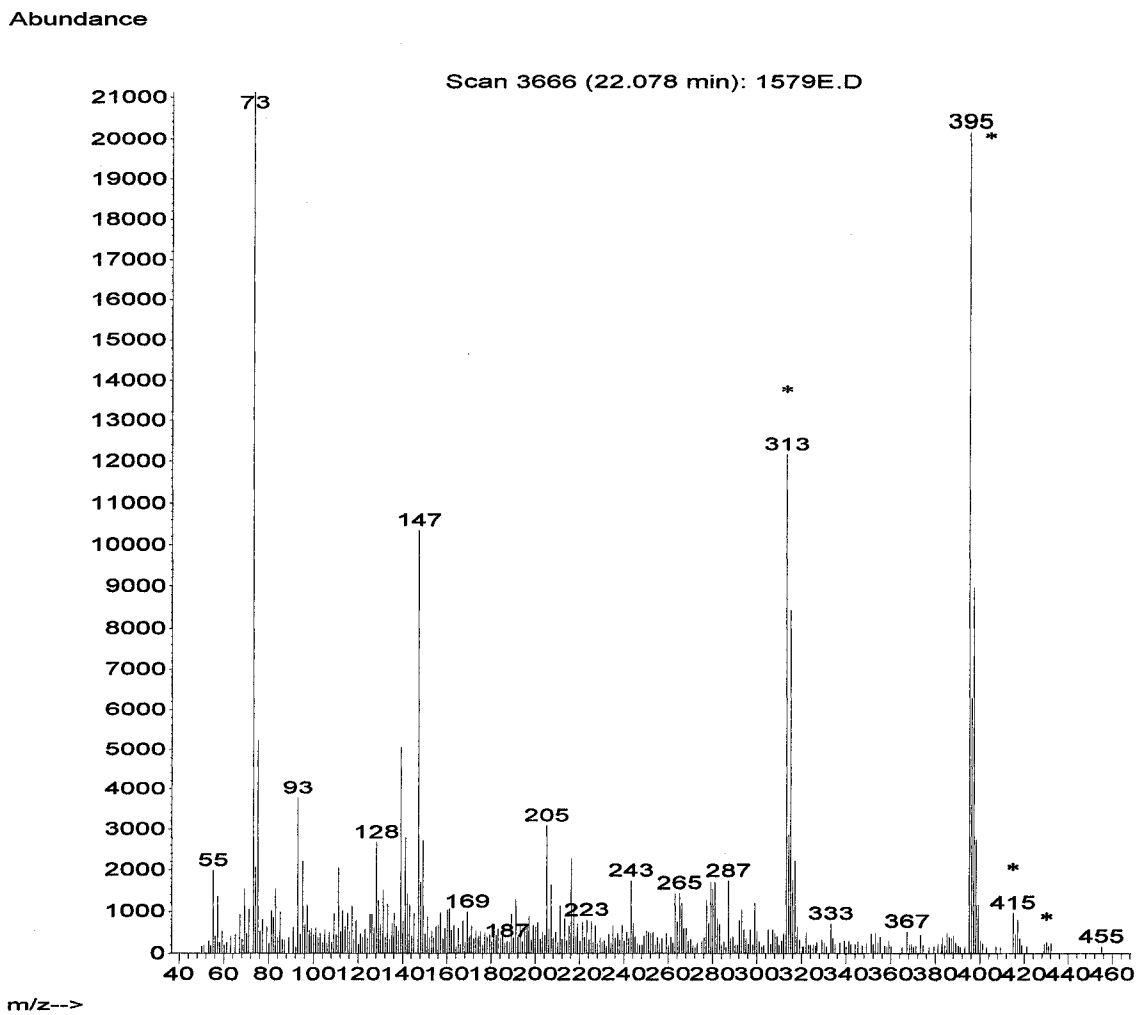
Mass spectrum of the compound in **peak no. 15** proposed to be dichloro-dihydrodiol



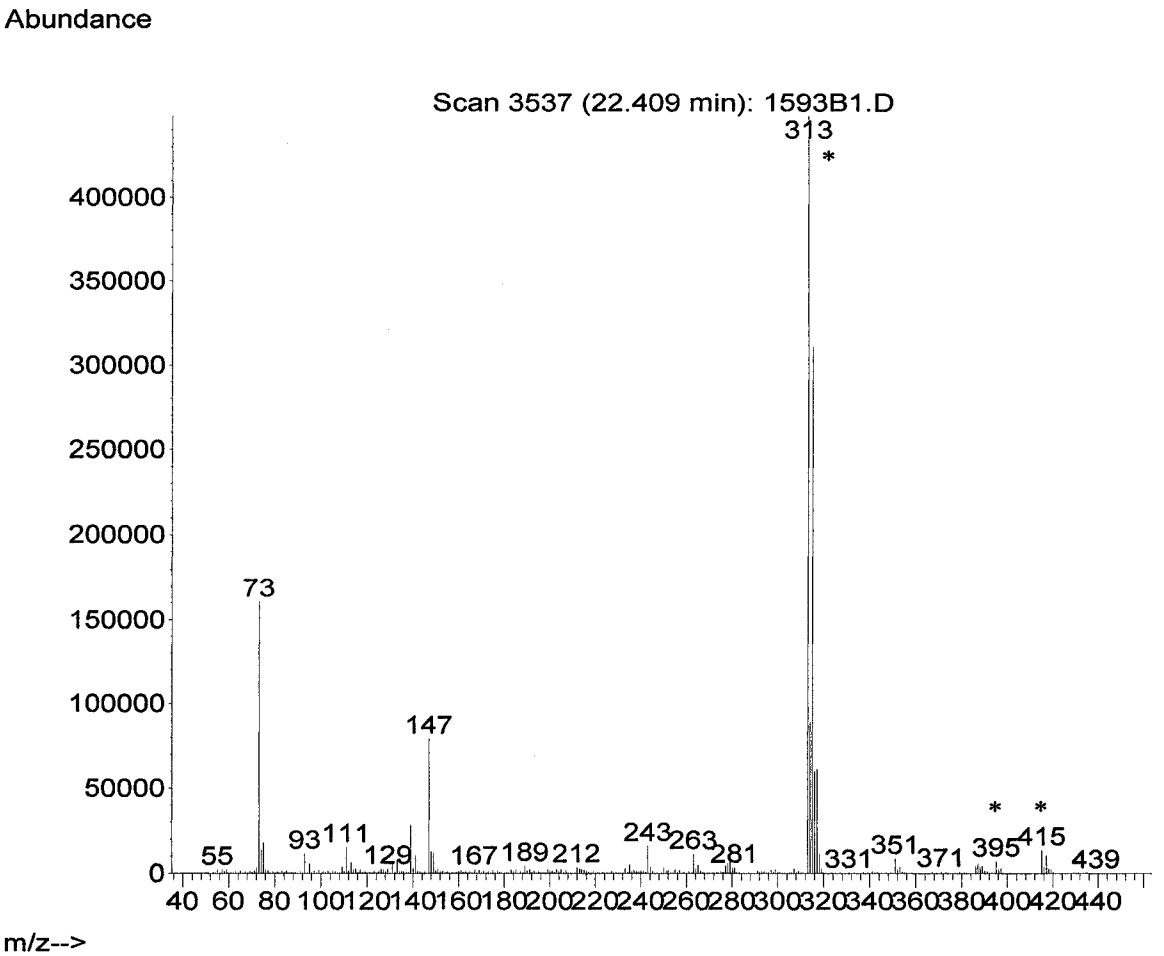
Mass spectrum of the compound in **peak no. 16** proposed to be dichlorodihydroxybiphenyl



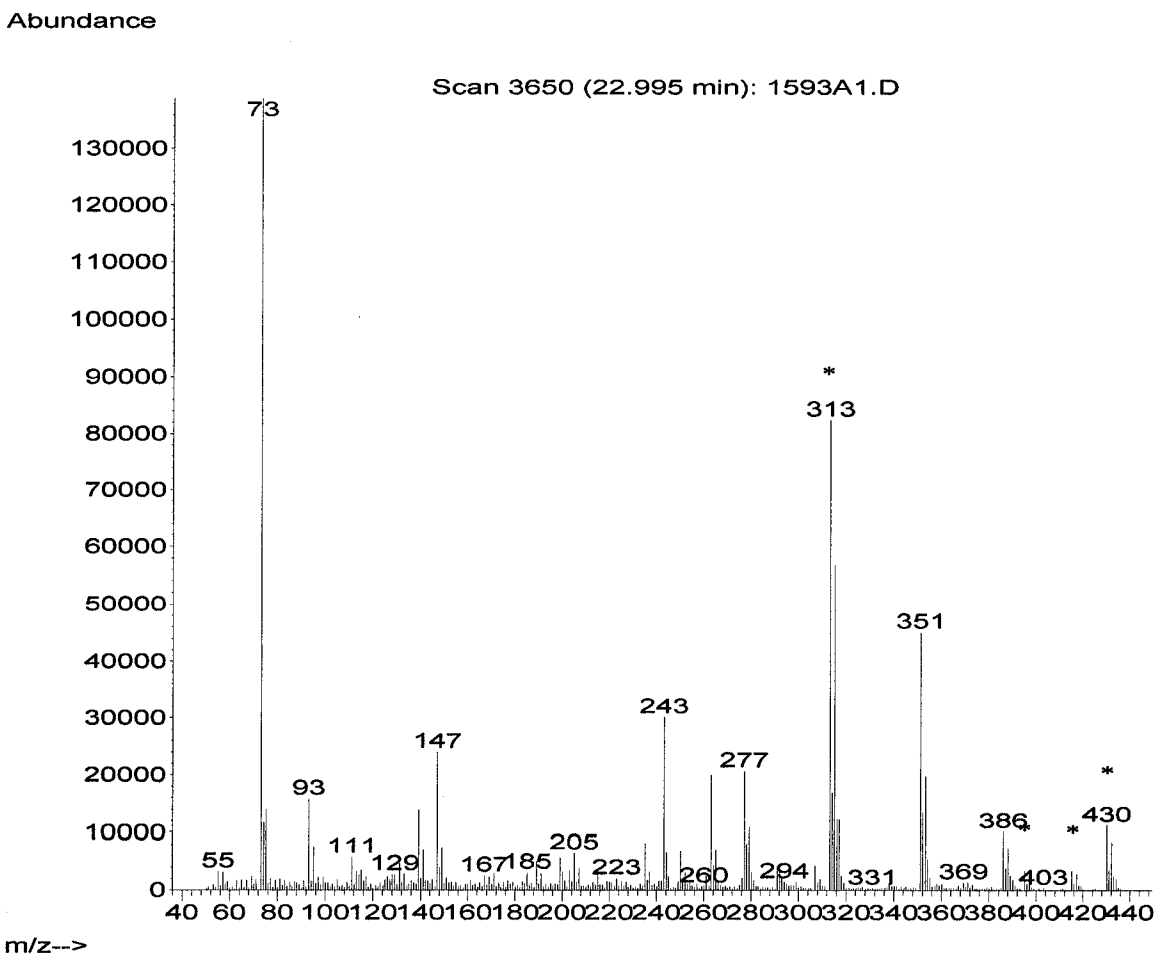
Mass spectrum of the compound in **peak no. 17** proposed to be dichlorinated HOPDA



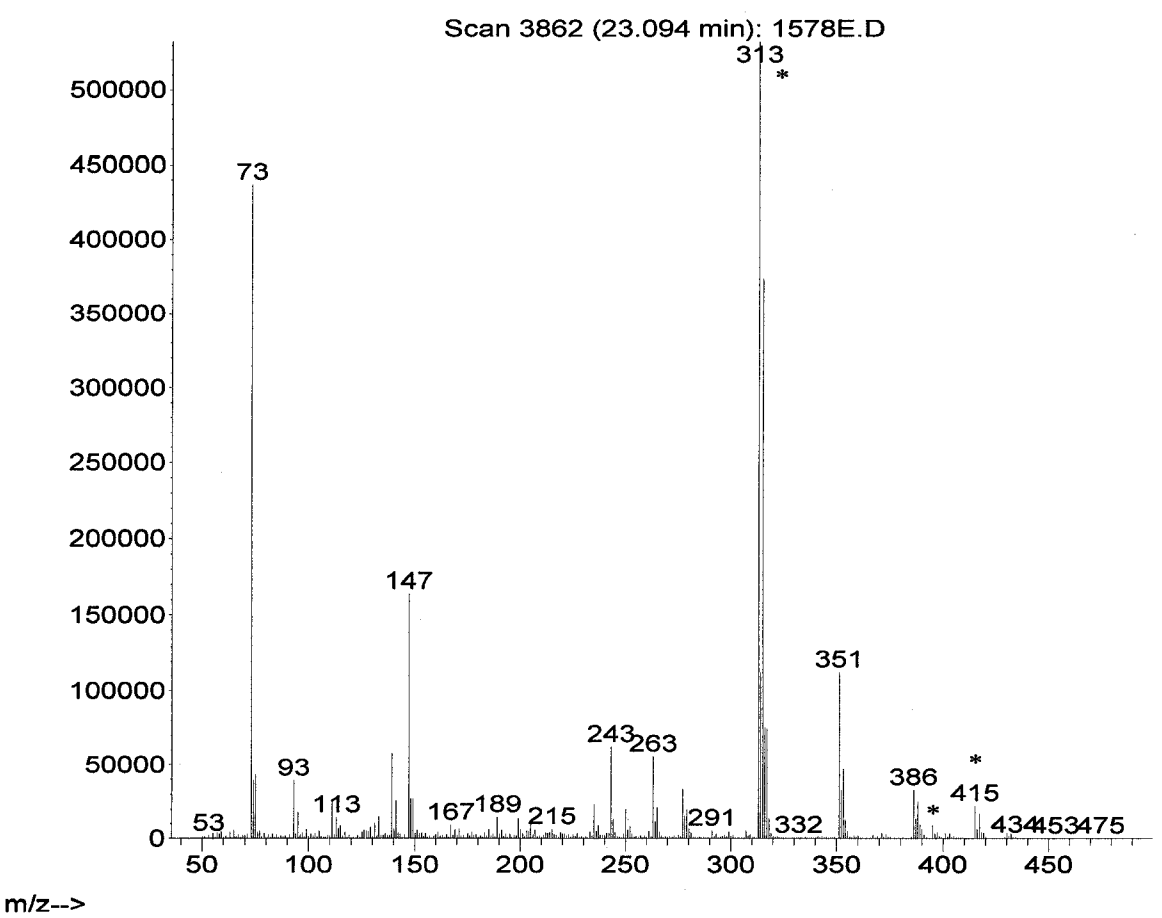
Mass spectrum of the compound in **peak no. 18** proposed to be dichlorinated HOPDA



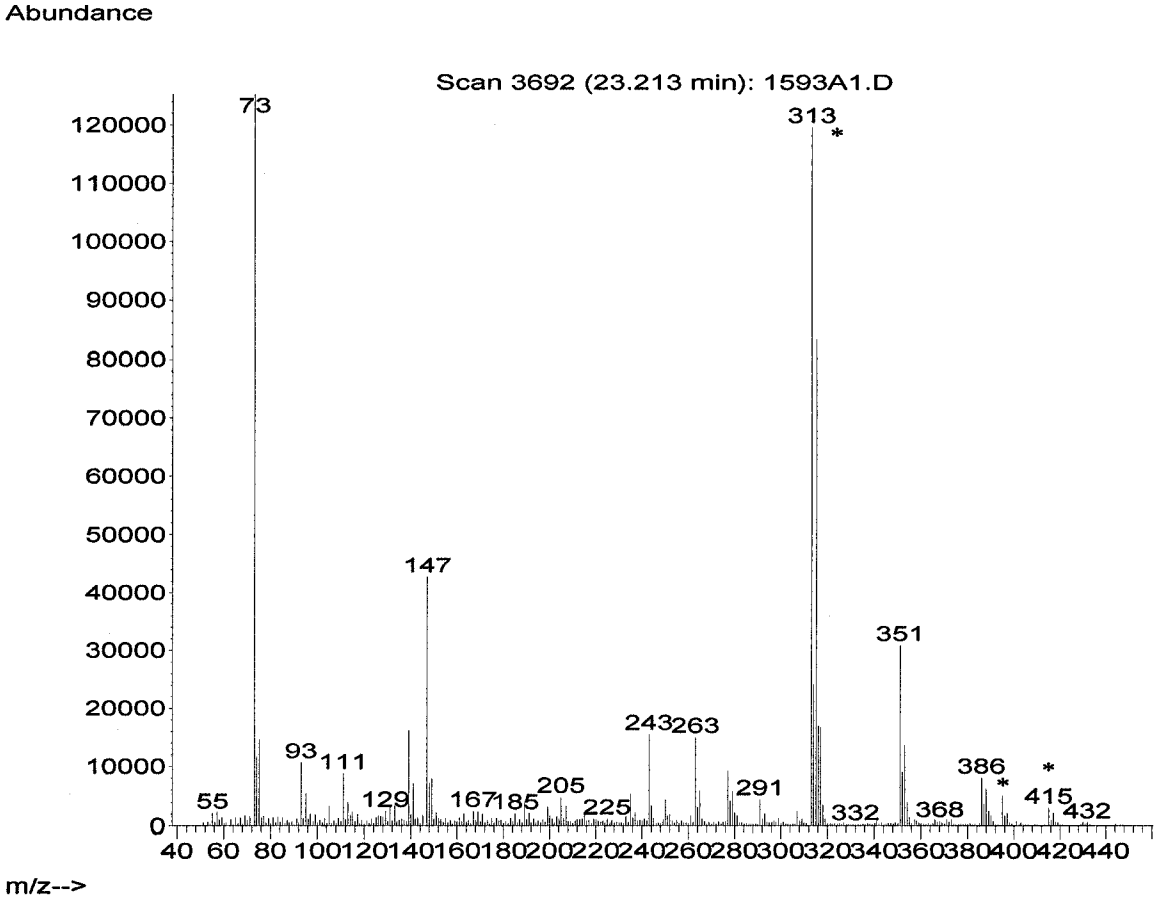
Mass spectrum of the compound in **peak no. 19** proposed to be dichlorinated HOPDA.



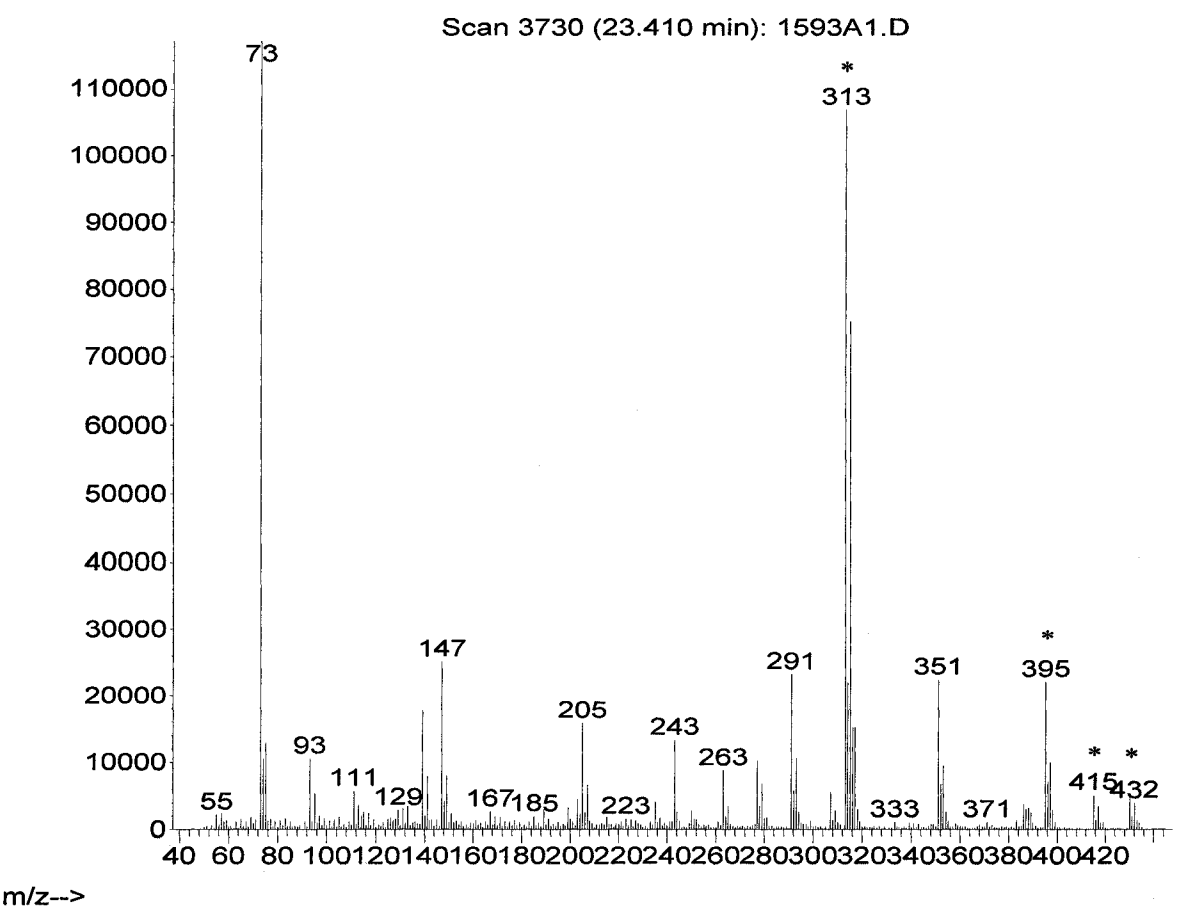
Mass spectrum of the compound in **peak no. 20** proposed to be dichlorinated HOPDA



Mass spectrum of the compound in **peak no. 21** proposed to be dichlorinated HOPDA



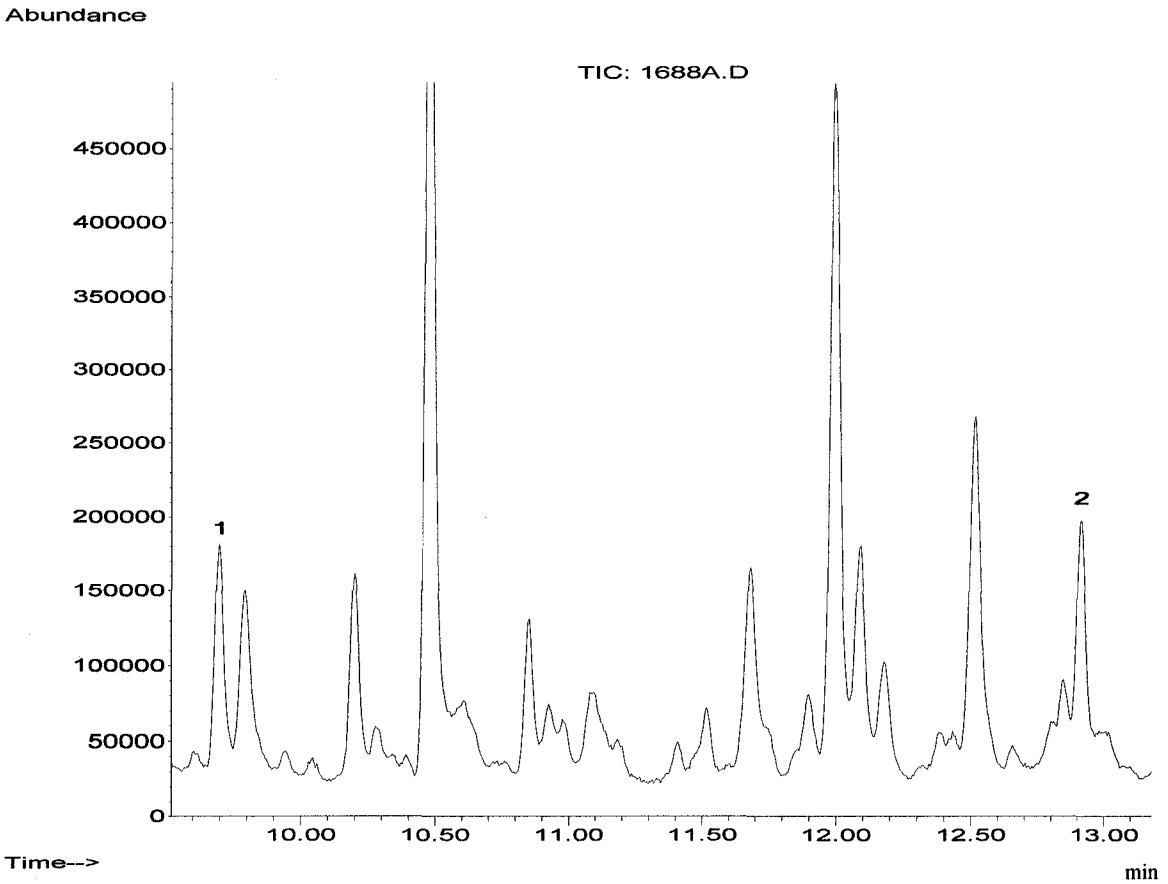
Mass spectrum of the compound in **peak no. 22** proposed to be dichlorinated HOPDA



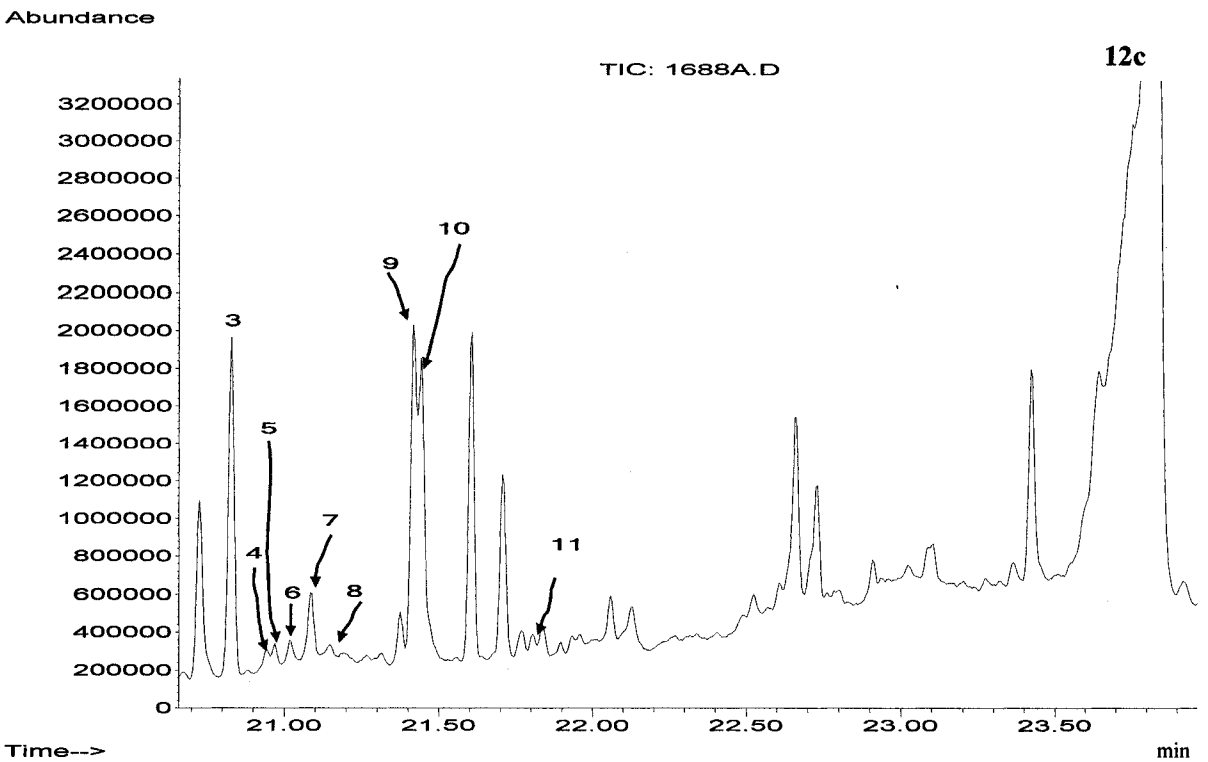
Mass spectrum of the compound in **peak no. 23** proposed to be dichlorinated HOPDA

Appendix C

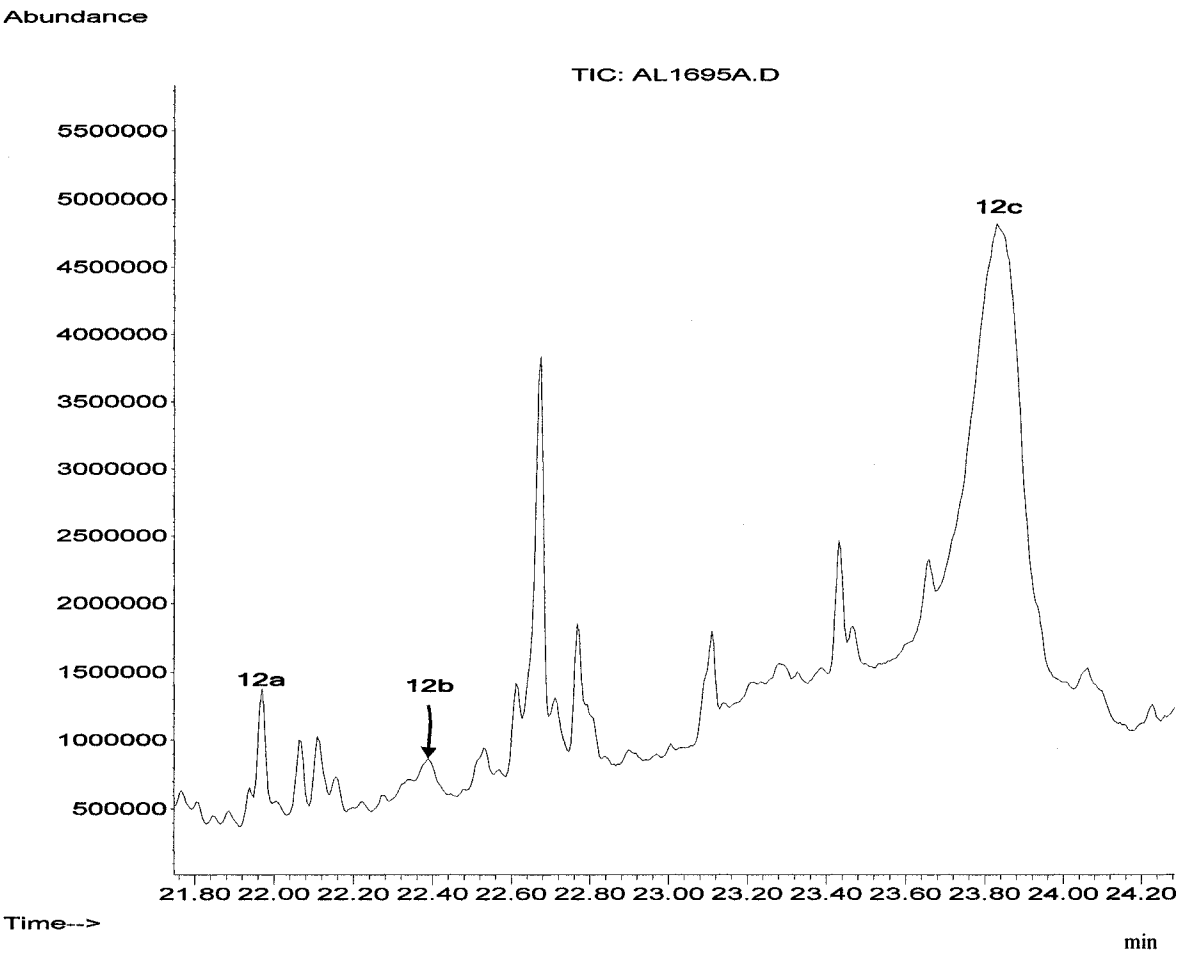
Gas chromatogram and mass spectra of TMS-derivatized metabolic products detected in acidic and neutral extracts of cultures of biphenyl-grown cells of *Hydrogenophaga* sp. IA3-A incubated with 2,4,4'-dichlorobiphenyl.



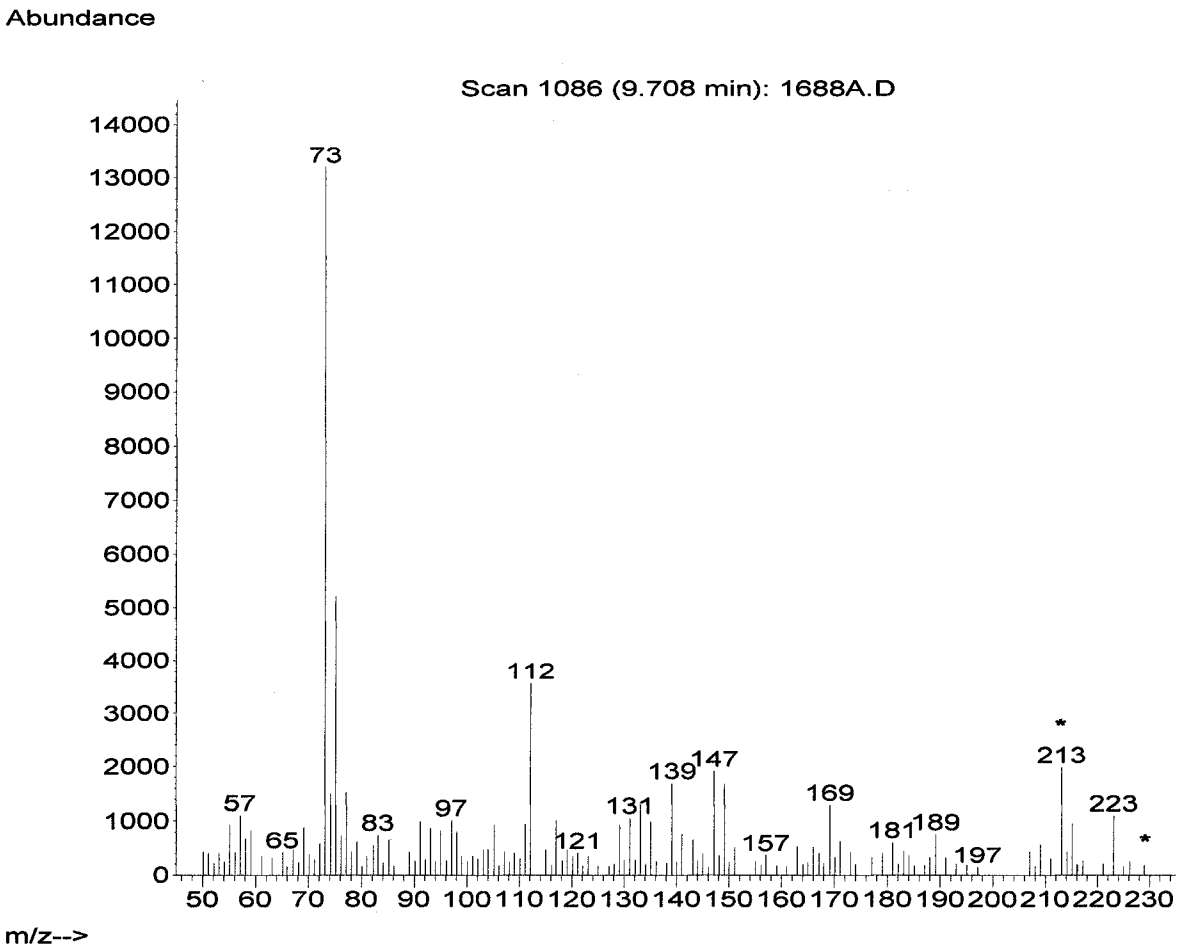
A section of the TIC of neutral and acid extract of cultures incubated with 2,4,4'-triCB at 5 °C h showing peaks corresponding to 4-CBA (peak no. 1) and 2,4'-CBA (peak no. 2).



A section of the TIC of neutral and acidic extracts of cultures incubated with 2,4,4'-TriCB at 5 °C for 72 h showing peaks with mass spectra corresponding to dichloro-dihydroxybi-phenyls (peak nos. 3 – 8), dichloro-dihydrodiols (peak nos. 9 – 11) and trichloro-HOPDA (peak nos. 12c). Mass spectra of the compounds are shown below.

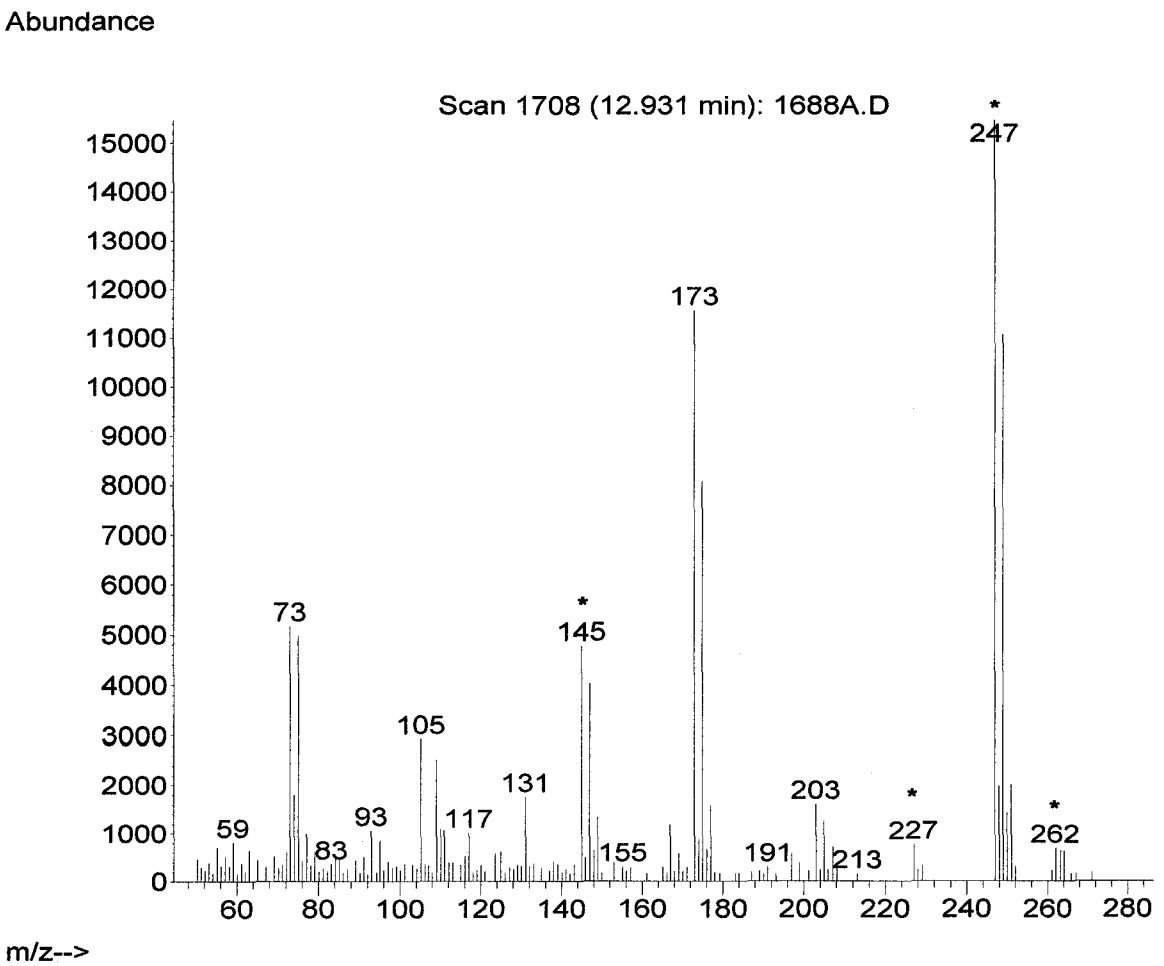


A section of the TIC of neutral and acidic extracts of cultures incubated with 2,4,4'-TriCB at 5 °C for 72 h showing peaks with mass spectra corresponding to trichloro-HOPDAs (12a – c). Note that all three compounds were only detected in one of the duplicate samples, and the TIC is shown above.

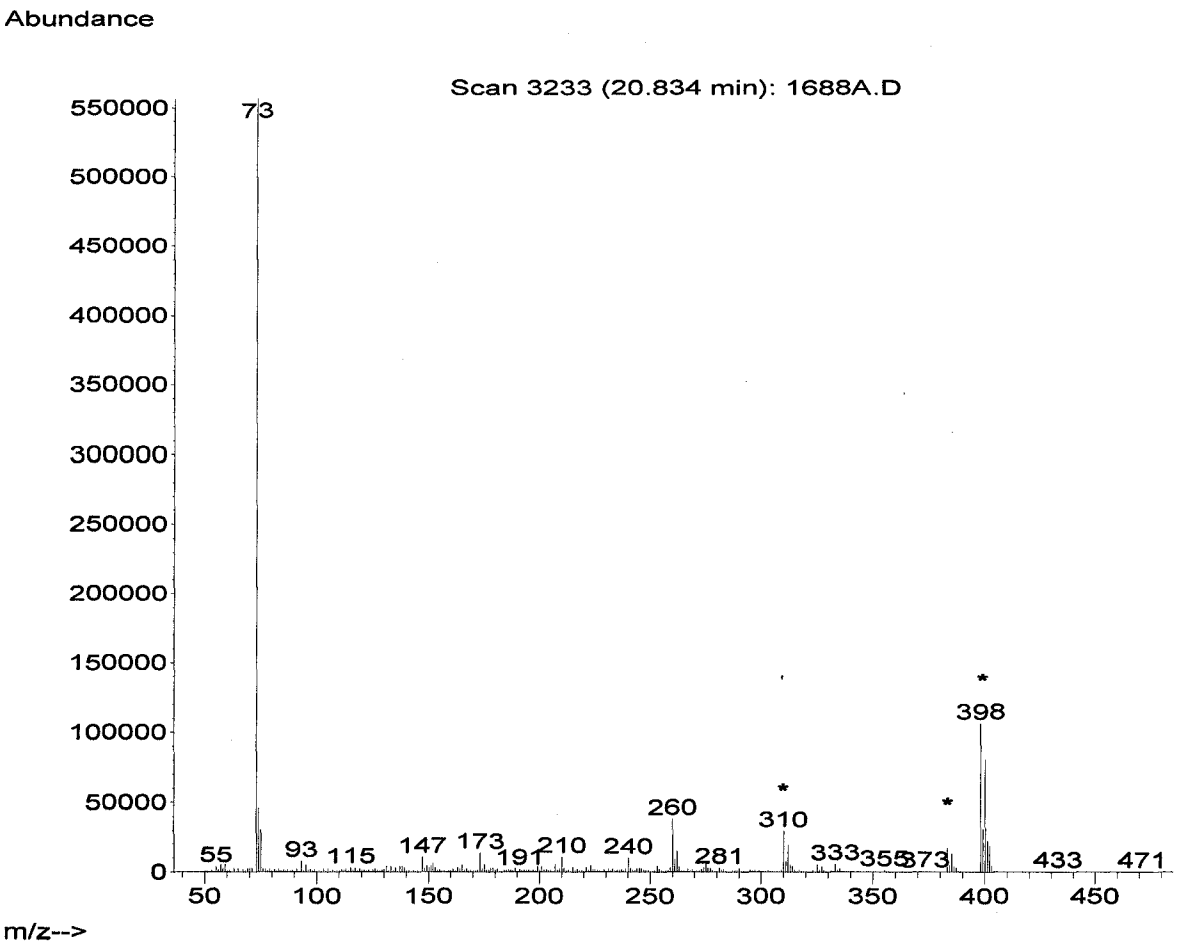


Mass spectrum of the compound in **peak no. 1** identified as 4-chlorobenzoate.

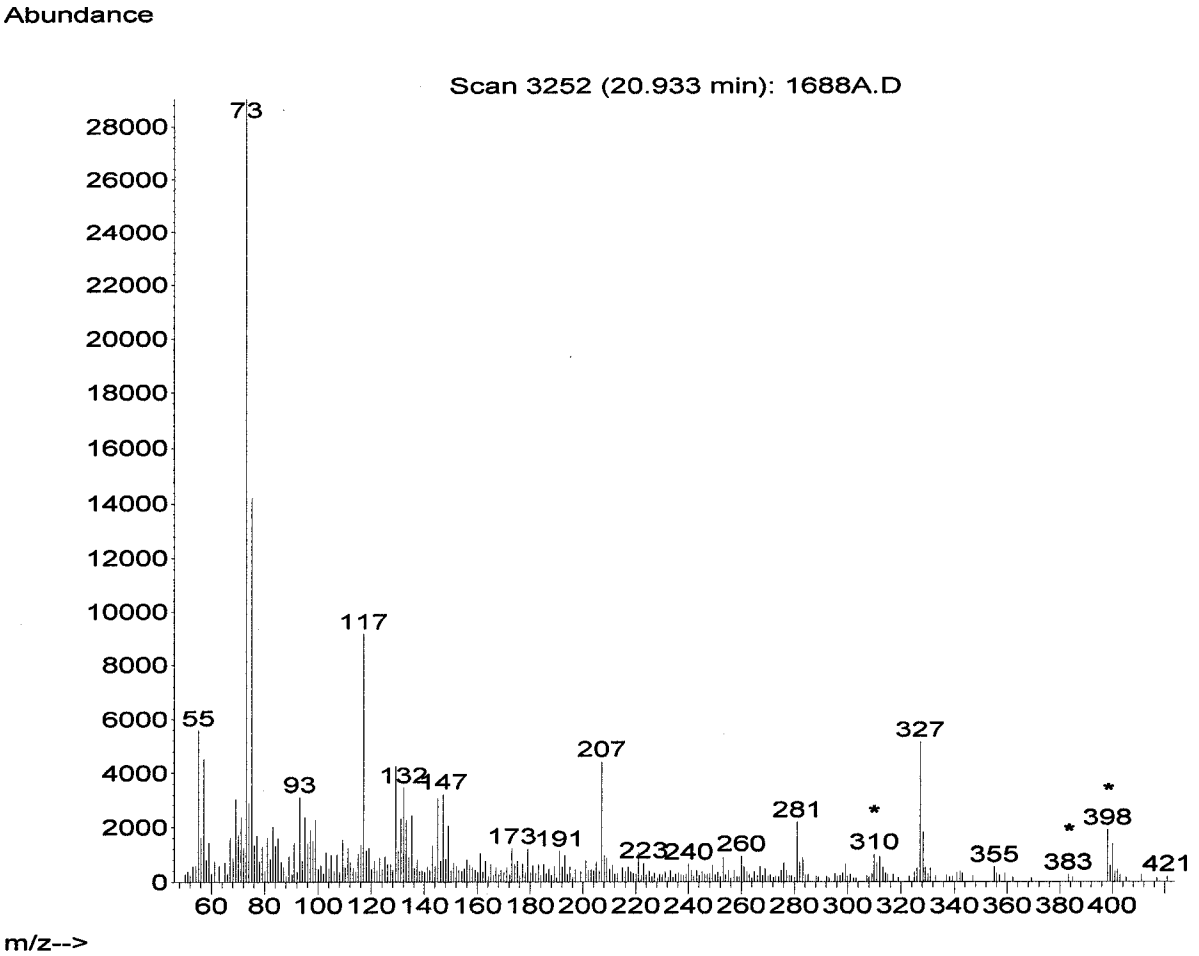
Characteristic ions are indicated with an asterisk (as shown below for other spectra).



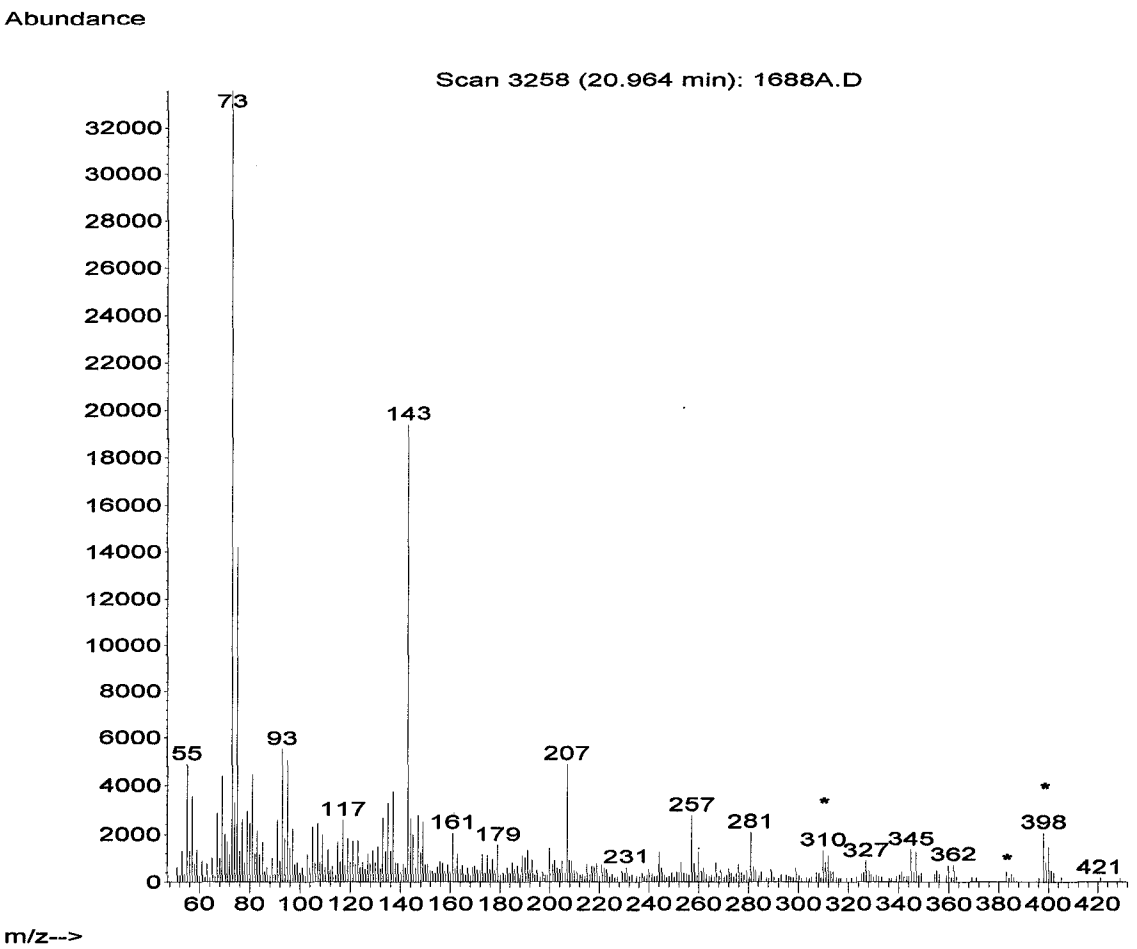
Mass spectrum of the compound in **peak no. 2** identified as 2,4-dichlorobenzoate



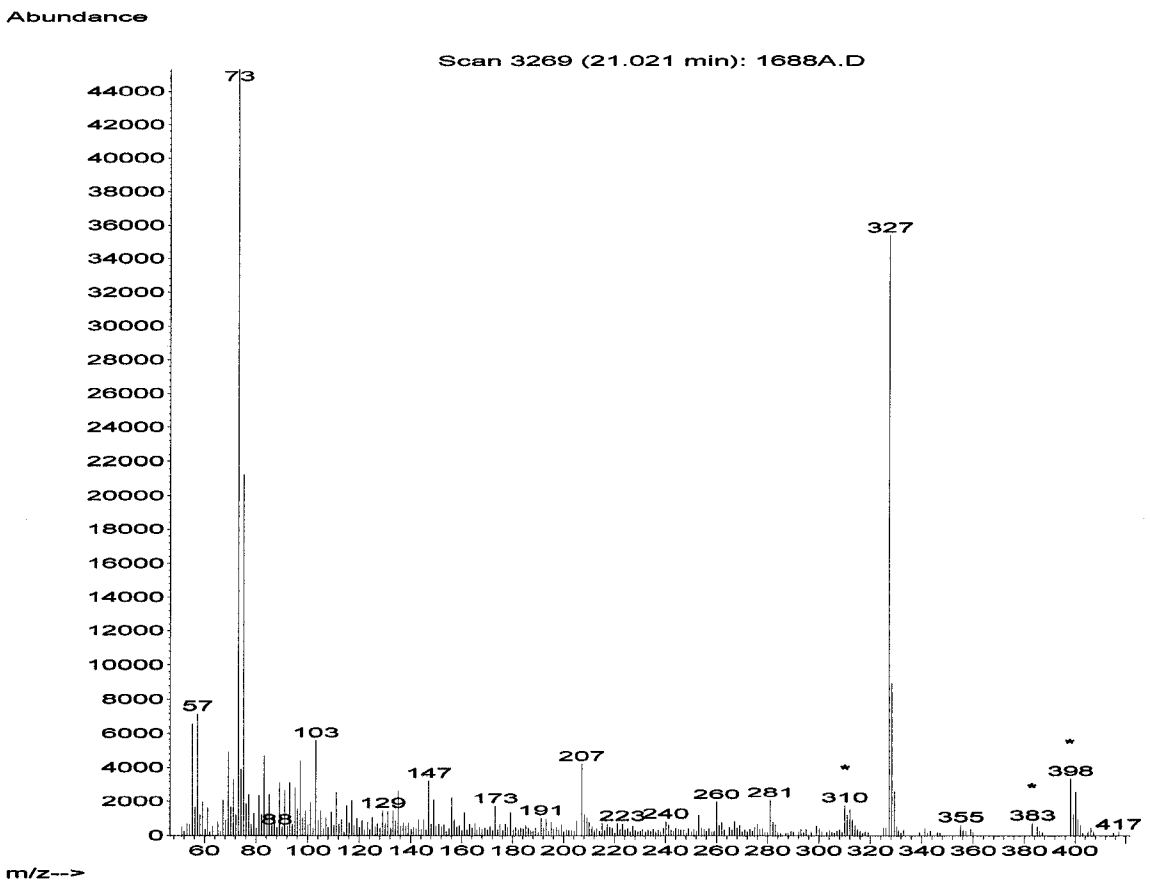
Mass spectrum of the compound in **peak no. 3** proposed to be dichloro-dihydroxybiphenyl



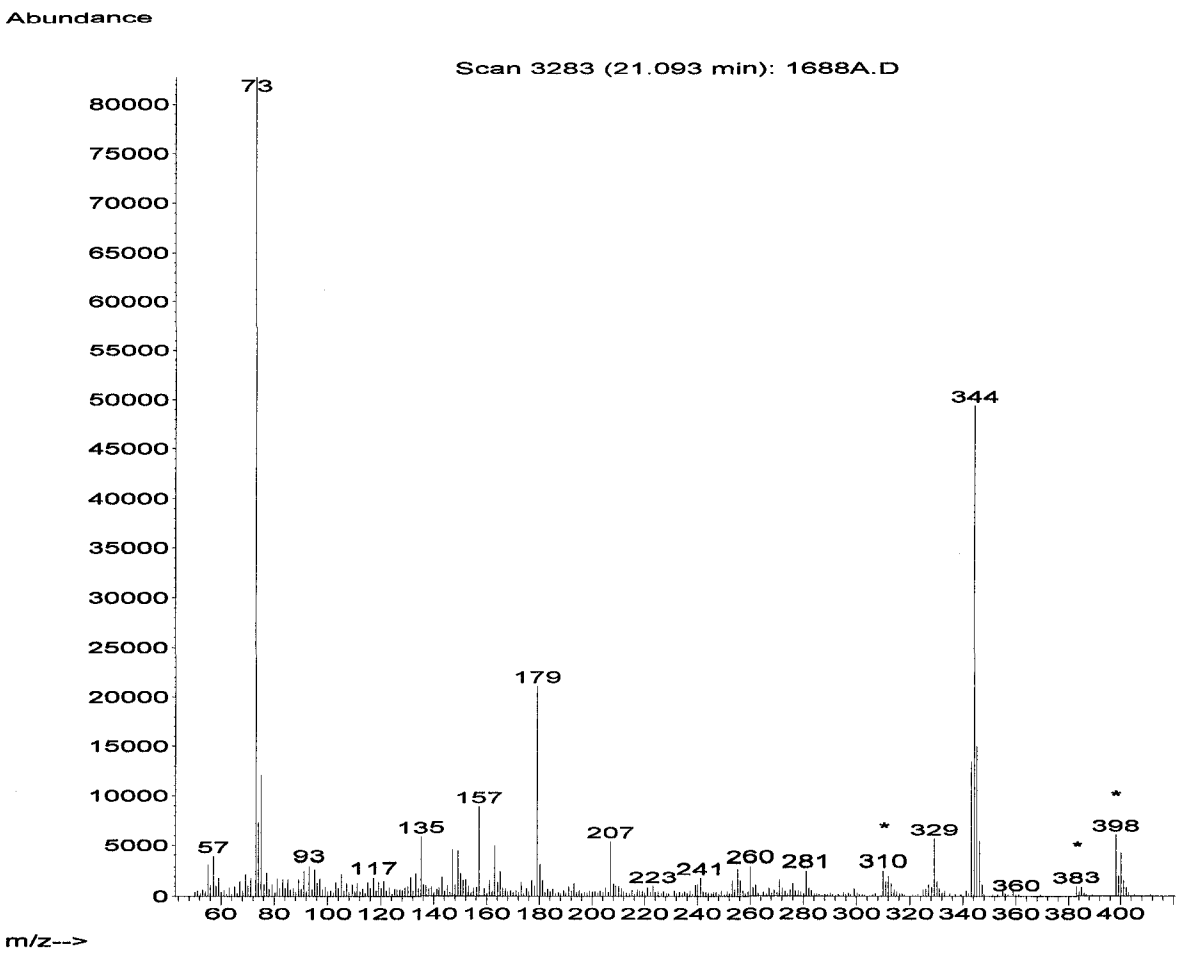
Mass spectrum of the compound in **peak no. 4** proposed to be dichloro-dihydroxybiphenyl



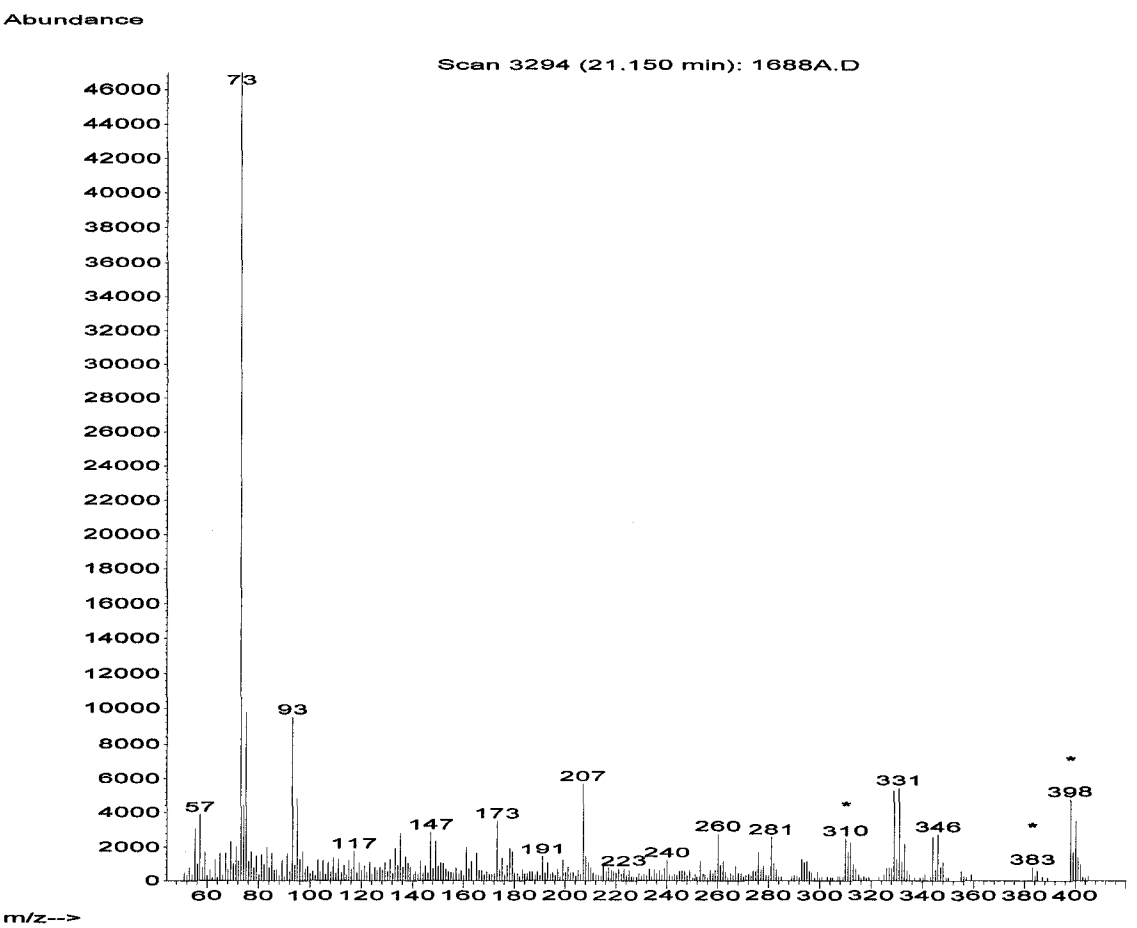
Mass spectrum of the compound in **peak no. 5** proposed to be dichloro-dihydroxybiphenyl



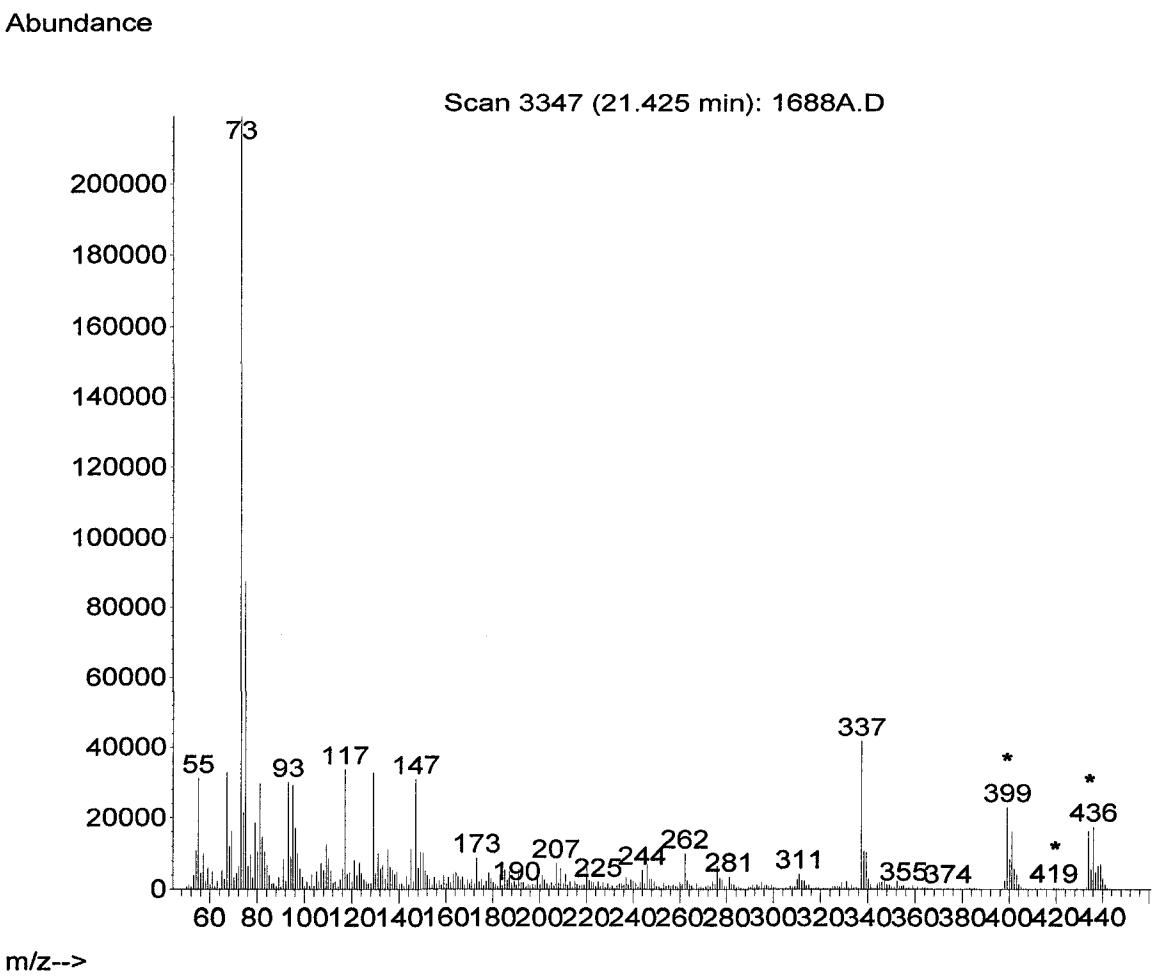
Mass spectrum of the compound in **peak no. 6** proposed to be dichloro-dihydroxybiphenyl



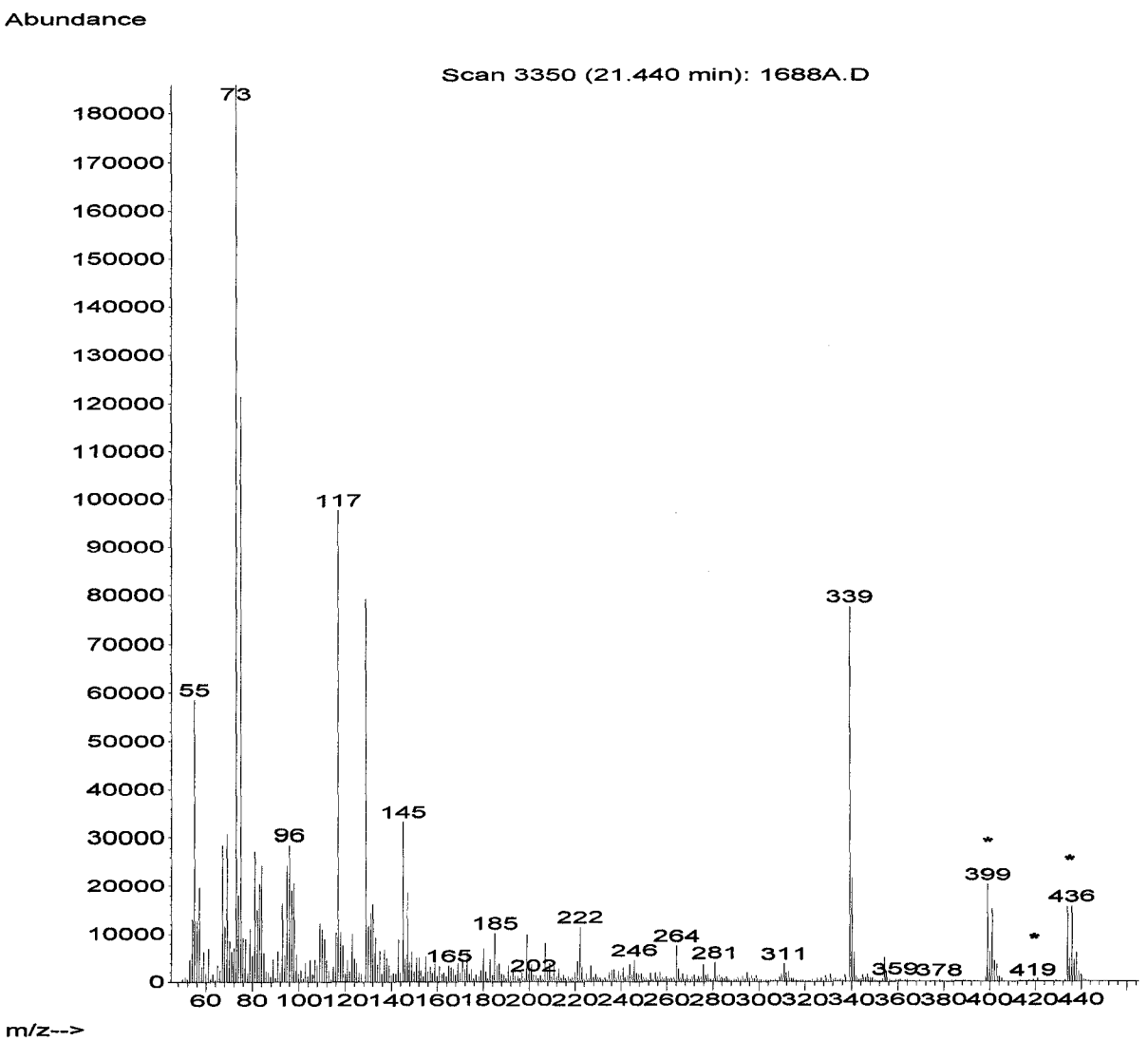
Mass spectrum of the compound in **peak no. 7** proposed to be dichloro-dihydroxybiphenyl



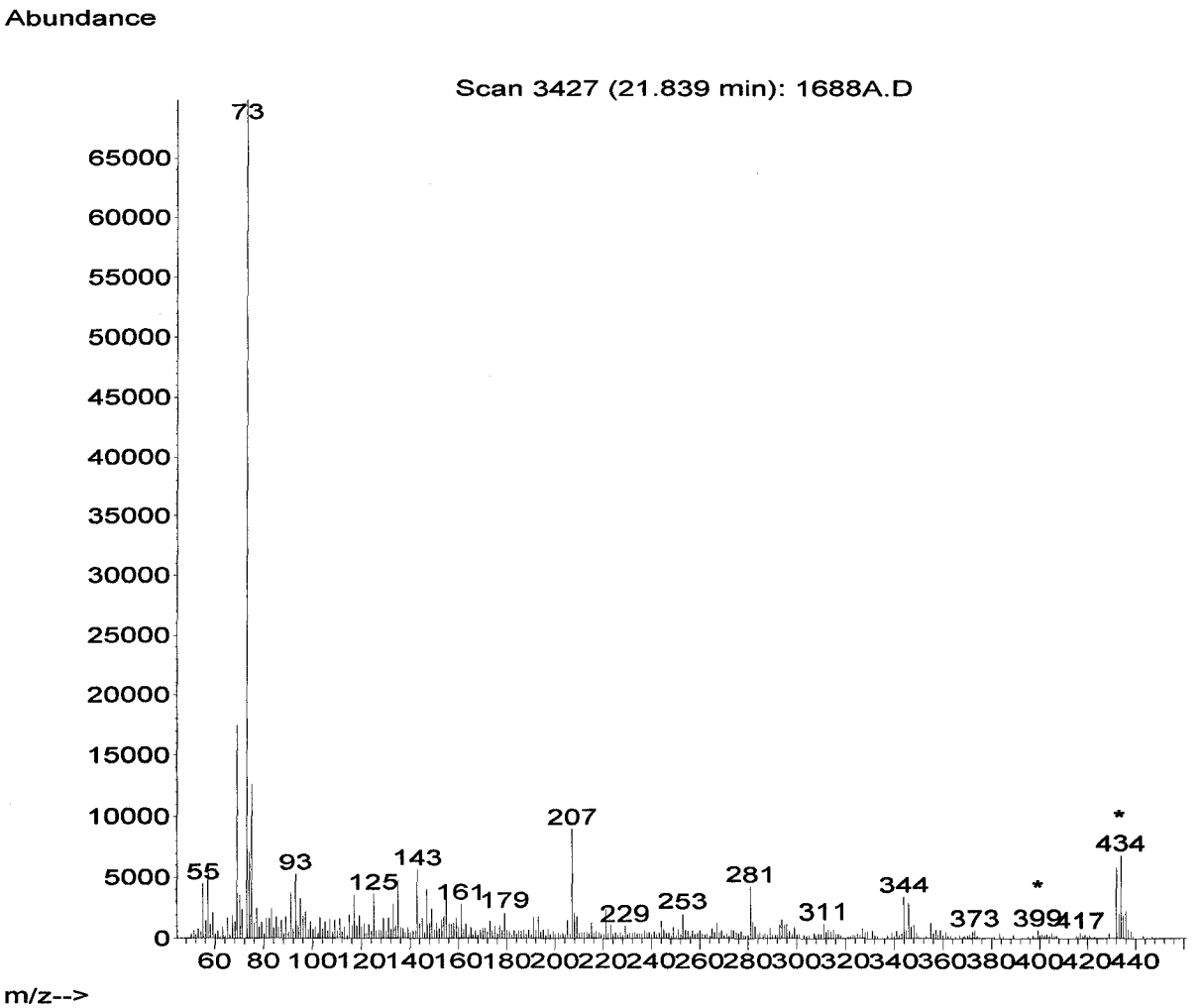
Mass spectrum of the compound in **peak no. 8** proposed to be dichloro-dihydroxybiphenyl



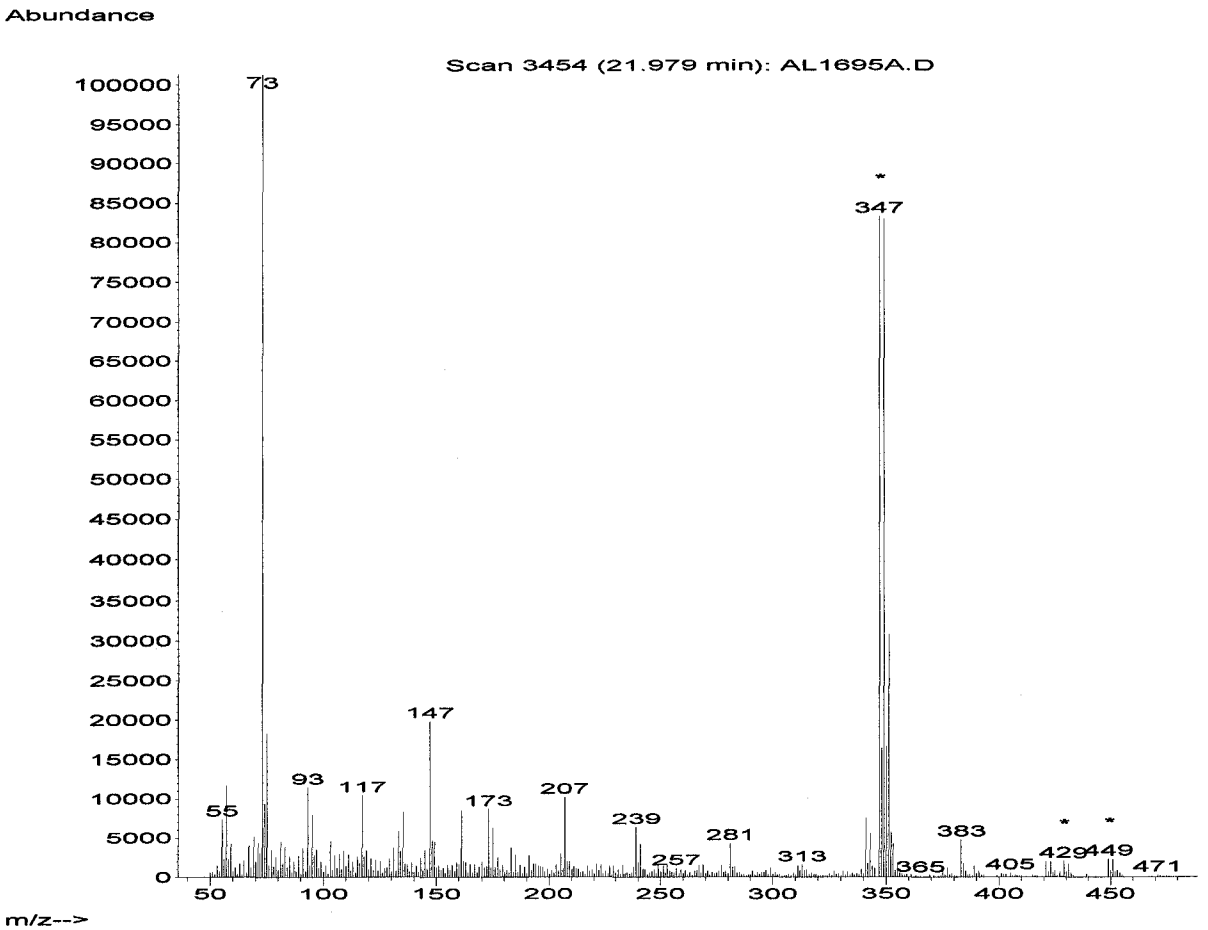
Mass spectrum of the compound in **peak no. 9** proposed to be 2,4,4'-dihydrodiol



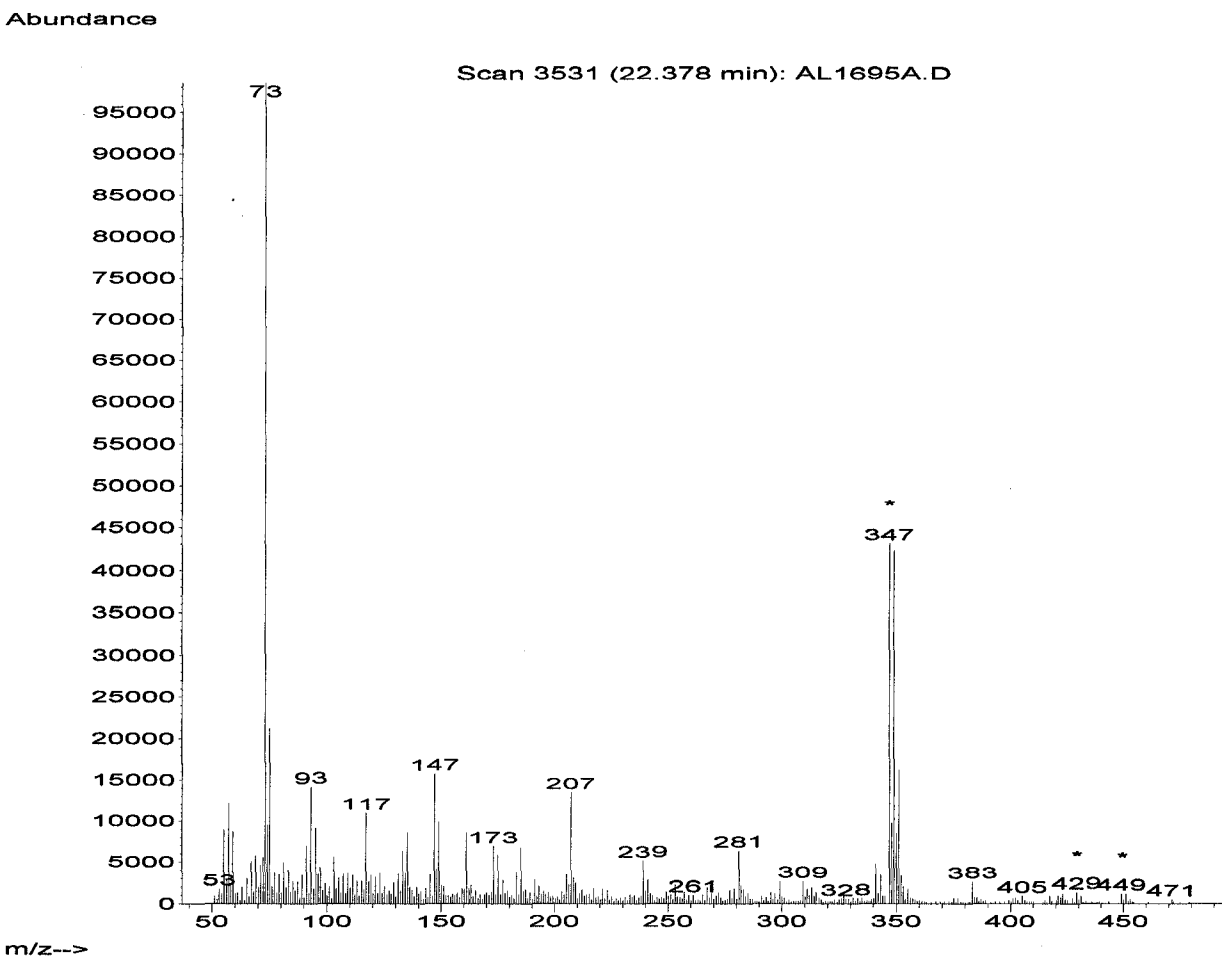
Mass spectrum of the compound in **peak no. 10** proposed to be 2,4,4'-dihydrodiol



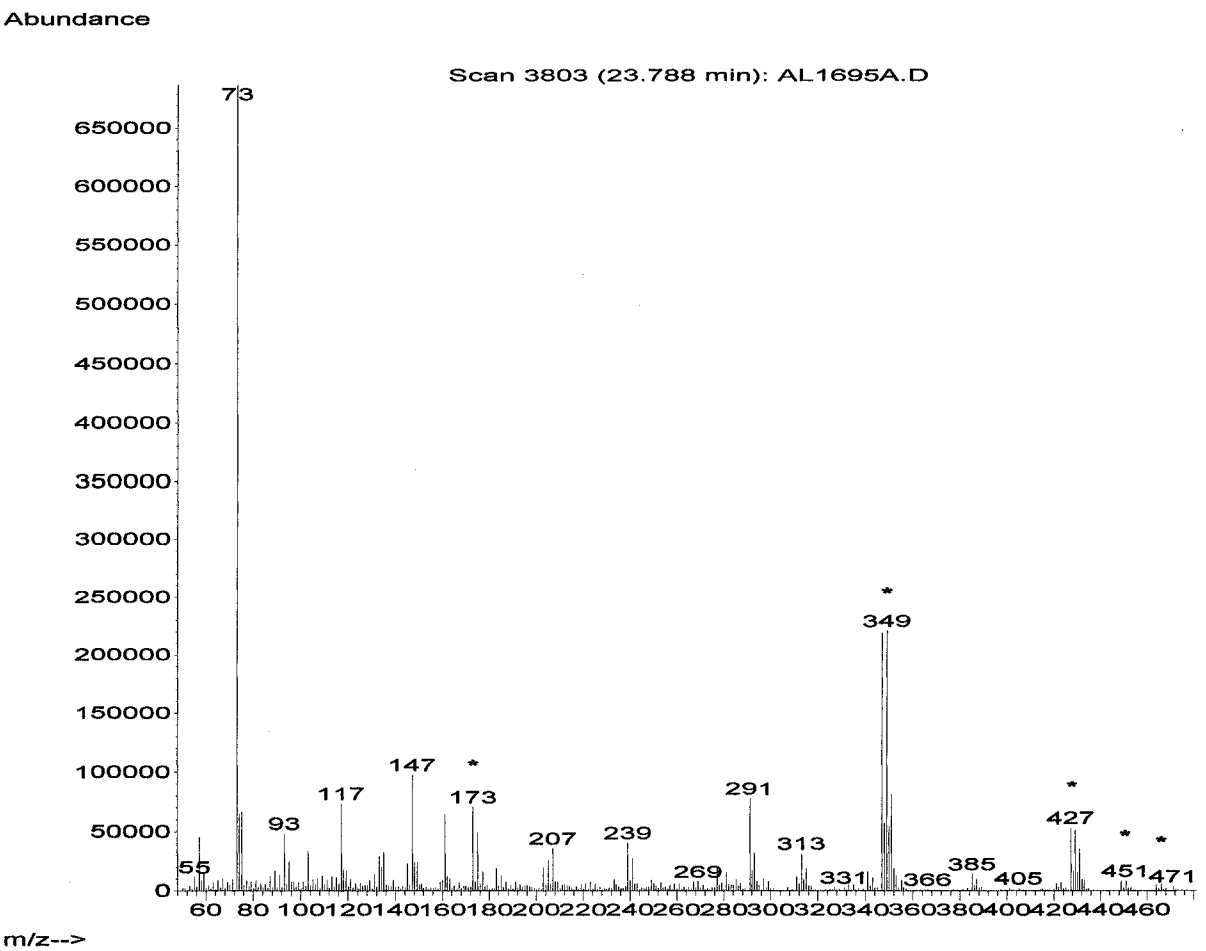
Mass spectrum of the compound in **peak no. 11** proposed to be 2,4,4'-dihydrodiol



Mass spectrum of the compound in **peak no. 12a** proposed to be trichlorinated HOPDA



Mass spectrum of the compound in **peak no. 12b** proposed to be trichlorinated HOPDA



Mass spectrum of the compound in **peak no. 12c** proposed to be trichlorinated HOPDA